A STUDY OF THE IMMUNOLOGIC RESPONSE TO SECOND HETEROTYPIC BLUETONGUE VIRUS INFECTION IN MICE

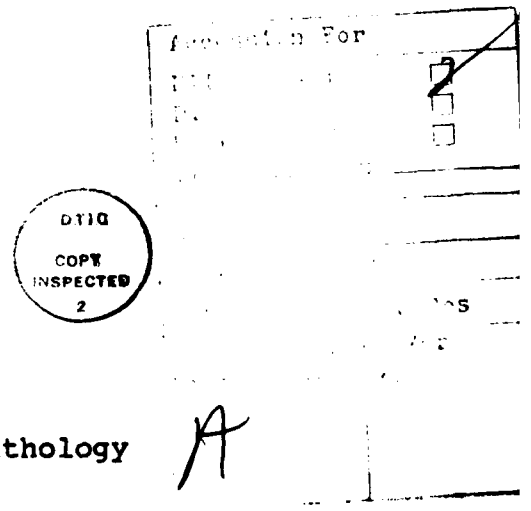
A Dissertation

by

WILLIAM MAX WITT

Submitted to the Graduate College of
Texas A&M University
in partial fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY



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ABSTRACT

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William Max Witt, A.A., Cisco Junior College;
B.S., D.V.M., Texas A&M University
Chairman of Advisory Committee: Dr. C. A. Holmberg

The first phase of the study involved the development of 2 immunological techniques, (1) an avidin/biotin-enhanced enzyme-linked immunosorbent assay (A/B-ELISA) and (2) an avidin/biotin-enhanced immunoperoxidase staining procedure, for the detection and measurement of serological responses of mice to bluetongue virus (BTV) infection and the demonstration of BTV antigen in mouse tissues. Results indicated that the A/B-ELISA is an effective assay for serum antibody produced against BTV in the mouse; and that BTV antigen is detectable in infected mouse tissues with the avidin/biotin-enhanced immunoperoxidase technique.

The 2 immunological techniques were utilized in the second phase of the study to investigate the immune response of adult mice to selected single and sequential BTV serotype infections, and to investigate the effect of prior passive immunization on the immune response of adult mice to subsequent BTV infection. Results indicated that the C57Bl/6 strain of mice responded with low antibody titers following a single BTV infection, but that titers

significantly increased following either sequential homotypic (same serotype) or heterotypic (different serotype) infections. It was further demonstrated that the immune response following heterotypic BTV infections was greater than that following homotypic infections.

These observations along with immunoabsorbent studies suggest that much of the response to heterotypic infections is directed against the secondarily infecting virus serotype. These results are in contrast with previous views, based largely on in vitro serum neutralization tests, that infection of an animal by 1 serotype of a virus should not alter the host immune response to infection by a different serotype of that virus.

Clinical signs of illness were not observed nor were histologic lesions recognized in any of the mice infected with BTV. Neither was specific immunoperoxidase staining observed in the tissues of adult experimental mice killed 14 days after the last virus infection. However, BTV antigen was readily demonstrable in positive control suckling mouse brain sections and in the hearts and spleens of adult control mice killed on days 5 and 6 following BTV infection.

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CHAPTER I

INTRODUCTION

Bluetongue virus (BTV) is an arthropod-borne agent known to infect sheep, cattle, goats and wild ruminant species with variable disease expression (20, 69, 70, 158). Bluetongue was first recognized and described as a distinct clinical entity in sheep in 1876 (3) and has been assumed to occur following arthropod transmission to susceptible ruminants. Clinical disease results from virus-induced cytopathic changes (23, 73). The disease has been well studied with numerous reports of both spontaneous and experimental ruminant BTV infections cited (13, 18, 19, 23, 24, 34, 38, 40, 47, 50, 52, 56, 62, 64, 71-73, 83, 91-95, 106, 108, 111, 125, 136, 146, 147, 157, 167) and reviewed (3, 23, 73). However, deficiencies still remain regarding the basic understanding of the virus-host interrelationship which have prevented development of effective control measures and hampered movement of ruminants and their products between countries of the world.

Direct economic losses in the United States from BTV infections result from decreased milk and wool production, reduction in body weight and condition, fetal losses due to abortions and/or malformations, and mortality. Possibly more significant indirectly are the costs of testing regulations imposed on livestock exporting countries,

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including the United States. The principal concern of bluetongue-free countries lies in the possibility of importing BTV infected livestock from enzootic areas. Variable disease expression and inapparent carrier states frequently occur with BTV infection in ruminants. Serological methods and virus isolation procedures for the detection of BTV infection must, therefore, be relied on to prevent the importation of infected animals. At present, the modified complement-fixation (MCF) (16) and the agar gel precipitin (AGP) (84) tests are the standard serotests used by countries as a judgment of BTV infection. However, results of a recent study (124) indicate that the sensitivity of AGP tests are inadequate to determine infection. In this study, AGP tests were negative for antibody in 43% of the cattle and 23% of the sheep from which BTV was isolated.

BTV infection is widespread in nature, although disease expression is observed only as limited outbreaks in certain herds or flocks (11, 20, 124). Several factors that might control disease expression in animals have been considered. It has been known for many years that various factors such as breed, individual animal susceptibility, environmental conditions, stress, and pathogenecity of the particular viral isolate influence the BTV-host interrelationship (117, 118, 157). Understanding this variability in disease expression resulting from BTV

infection will depend on defining the pathogenesis of disease induction. There remains a dispute in the literature as to the tropism of BTV. The virus has been reported to have an affinity for epithelial (23, 73), reticuloendothelial (2, 174), endothelial (147, 148), and periendothelial (147, 148) cells. One pathogenesis study in sheep proposed that following subcutaneous inoculation, the virus was first associated with macrophages and lymphocytes in the regional lymph nodes, and then spread by way of the lymph and blood to lymphoid tissues in other parts of the body where further replication occurred (132).

Genetic variation of the virus has also been considered as a possible reason for variable disease expression. The BTV genome is composed of 10 segments of double-stranded ribonucleic acid (RNA). The possibility exists that simultaneous infection of a susceptible animal with multiple BTV serotypes could result in the formation of viral recombinants. Such recombinants could be antigenically different and, therefore, might not be neutralized by the animal's immune response. With replication of a viral recombinant, the severity of virus-induced lesions could likewise be altered. The existence of viral recombinants was first proposed when the inclusion of more than 4 strains of BTV in a single modified live virus vaccine appeared to induce, rather than afford protection against, disease (CA Holmberg, personal

communication). There is a possibility that viral recombinants exist in the United States since multiple BTV serotypes have been isolated from several infected herds of cattle and flocks of sheep (10, 124, 125).

An additional mechanism which would influence disease expression would involve an immunologic modulation that might occur following infection by 2 or more different viral serotypes. This mechanism has been proposed to explain the hemorrhagic death syndrome associated with sequential dengue virus strain infections in humans (60), and the fatal systemic disease in cats sequentially infected with feline infectious peritonitis coronavirus (171). In previous studies on the pathogenesis of bluetongue in sheep (148), it was shown that following experimental BTV inoculation of susceptible sheep, development of clinical signs and lesions followed the maximal virus infection of cells by several days and coincided with the occurrence of significant levels of antibody. This observation suggests that the host's immune response may play a significant role in the development of lesions associated with BTV infections. In addition, sequential infection with 2 or more serotypes of BTV may modulate such immunologic response so that lesion severity and ultimately disease expression can be variable.

Study of the immunologic responses of an animal to different BTV serotype infections would provide a better

understanding of the BTV-host interrelationship. Although sheep are recognized as the conventional experimental species for studies of bluetongue (122), they along with cattle, are difficult to utilize in basic BTV research due to their initial cost, the expense of long term maintenance, and the difficulty of obtaining susceptible, non-immune animals. Also, one of the more basic, yet little publicized, problems associated with experimental BTV infections in sheep is the inability to consistently induce a disease that approaches the severity and nature of the spontaneous one. It would, therefore, be difficult to study basic immunologic responses of animals to different BTV serotype infections utilizing sheep or cattle as the host animal. However, much of the early pioneering, as well as current research providing basic knowledge and understanding of immunologic responses, has been accomplished with murine model systems employing genetically defined inbred strains of mice. Such animals are comparatively inexpensive to purchase and maintain so that large numbers can be utilized. Even though the mouse-BTV infection model has not been considered an effective model system for the study of typical ruminant bluetongue disease (81, 82, 107, 119), it has been utilized to study the effects of viral infection on the development of the nervous system (116), the kenetics of antibody production in single serotype infections (107, 119-122),

and the induction of cytotoxic T-lymphocytes (81, 82); and therefore, should be an advantageous system for the study of immunologic responses to sequential heterotypic, i.e., different serotype, BTV infections.

CHAPTER II

REVIEW OF THE LITERATURE

Bluetongue virus (BTV) is presently classified within the genus Orbivirus, family Reoviridae (15, 25, 127, 165). Although related to reovirus, orbiviruses are smaller in size, do not have a clearly structured outer capsid layer, are more resistent to lipid solvents, more labile at low pH and high temperature, and require an intermediate insect host for their transmission to mammalian hosts in nature (165). Bluetongue virus contains a segmented genome consisting of 10 double-stranded molecules of RNA and has an estimated total molecular weight of 6.0×10^7 daltons (165). The molecular weights of certain BTV genomic segments correlate exceptionally well with certain capsid polypeptides which suggests a possible coding function for those segments (164). The virion diameter has been reported to range from 54 to 75 nm depending upon the method used for purification (41, 112, 164). Complete BTV particles were demonstrated to have a double-layered capsid composed of a well defined inner layer consisting of 5 polypeptides arranged in icosahedral symmetry with 32 capsomers and a triangulation number T-3 (21, 22, 41, 112), and a diffuse outer layer comprised of 2 polypeptides designated P2 and P6. The diffuse outer layer has been reported to be essential for BTV infectivity and is considered to be coded for by the viral genome (104, 164).

In addition, this layer has been reported to give serological specificity (39, 79) to the various BTV serotypes with P2 being considered the dominant determinant (79). Bluetongue virus characteristically replicates in perinuclear cytoplasm that is recognized as viral inclusions or matrices (165). Maturing virions have been associated with fine cytoplasmic filaments and tubules of similar or smaller diameter than that of the virus (165). Mature virions are liberated from the host cell following cell lysis. The possibility of the virus acquiring a membranous "pseudoenvelope" during extrusion has been suggested by observing particles surrounded by a membrane under certain conditions of specimen preparation (21, 90).

Currently there are 20 serotypes of BTV recognized on a worldwide basis. Four, BTV 10, BTV 11, BTV 13 and BTV 17, are present in the United States with 1, BTV 17, considered to be unique to North America (10). There is serological cross-reactivity between BTV and epizootic hemorrhagic disease virus (EHDV) with the modified complement-fixation (MCF) and agar gel precipitin (AGP) assays. The latter virus is also classified as an orbivirus and infects ruminant species in the United States and Canada (165). Although the 2 viruses share many biochemical and biophysical properties, they are considered separate virus species (46, 165).

Bluetongue was first recognized as a separate clinical

entity in Africa in 1876 (3) following its occurrence in imported flocks of highly susceptible European breeds of sheep (23, 56). The disease remained confined to the African continent until 1943 when it was recognized in Cyprus and Turkey (4). Since that time, BTV has been known to cause infection and disease in ruminant species on a worldwide basis (19, 20, 34, 69, 165). In the United States, bluetongue was first suspected in Texas as early as 1948 (107) even though the first reported cases, originally called "sore muzzle," were not reported until 1951 (62). A syndrome resembling, and later confirmed to be, bluetongue was described in California in 1953 (108). Serological evidence now indicates that within the United States, BTV has a wide geographic distribution and a diverse host population that includes domestic and wild ruminants as well as insect vectors (11, 124). With the recent isolation and identification of BTV from an animal in New York state, the number of states from which BTV has been isolated has increased to 30 (11).

Early researchers considered bluetongue to be primarily a disease of sheep with a less frequent involvement of cattle and goats (44, 45, 69, 70, 73). However, more recent findings have indicated that the natural host range of BTV is broad and includes many ruminant species, most of which express variable disease manifestations following infection (20, 36, 66, 67, 69, 70,

158). Nonetheless, BTV infection results in clinically recognized illness more commonly in sheep that it does in cattle and other ruminants. The disease, in sheep in the United States, is less severe (mortality less than 10%) than that which affects the same species in Africa (mortality often approaches 90% with certain BTV strains) (107). Such differences in disease expression have been explained by factors such as breed, host susceptibility, environmental conditions, stress, and pathogenicity of the particular viral isolate, as influencing the BTV-host interrelationship (118, 157, 161).

Characteristic clinical signs and lesions of bluetongue in sheep include pyrexia, nasal discharge, hyperemeia, edema and congestion of the muzzle, tongue, lips and ears, shallow ulcers in the labial and gigival mucosa; anorexia, depression and initial stiffness that tends to disappear with exercise (18, 50, 91). A less consistent and later developing lesion is lameness associated with inflammation of the coronary band (50, 111, 157) and the postural skeletal muscle (111). Cyanosis of the tongue, the characteristic lesion for which the disease was named, is generally not a feature of the disease as it occurs in the United States. Clinical signs and lesions are infrequently observed in cattle in either experimental or naturally occurring infections (16, 19, 50), although in occasional field cases, the clinical signs

and lesions are similar to those reported for sheep and deer (13, 20). Goats and antelope rarely develop clinical signs or macroscopic lesions (72, 146). Neutropenia, lymphopenia, eosinopenia, and hemolytic anemia have occasionally been reported to occur with BTV infection in several species (49, 51, 91). The possibility that many of the clinical signs and gross lesions are secondary events following vascular damage has been recognized for several years (13, 111, 157). However, it was not until the pathogenesis of bluetongue in sheep was investigated using immunofluorescence (148) that the primary site of viral replication was demonstrated to be in the capillary endothelium. The vascular lesions were characterized by changes in endothelial cells that included nuclear and cytoplasmic hypertrophy, cytoplasmic vesiculation, and nuclear pyknosis and karyorrhexis that were accompanied by engorgement of vessels with blood and pericapillary hemorrhage and edema (148). In older lesions regenerative hyperplasia of capillary endothelium was also detected, and the lesions were more severe in the papillary connective tissue underlying stratified squamous epithelium of the oral mucosa, skin, and digestive tract (148). Vascular thrombosis, considered a significant lesion in experimentally induced disease in deer (89), has not been described in either sheep or cattle, but is a feature of the neurologic disease resulting from experimental BTV

inoculation of neonatal mice (107).

Encephalopathies, fetal death, and abortion have been reported in lambs and calves resulting from artificial or natural BTV infection of pregnant females with either virulent virus or attenuated vaccine strains (33, 54, 123, 134, 141, 174). There were reports of losses in newborn lambs due to developmental abnormalities in the fetuses of ewes vaccinated during the 5th and 6th weeks of gestation after egg-attenuated, modified live virus vaccine was released for field use in California in 1954 (141). Following full term pregnancy, some of the lambs were stillborn. Of the lambs that were born alive, many exhibited spasticity or head pressing and were blind or walked aimlessly. These lambs were often called "dummies" or "crazy lambs," and were found to have encephalopathies ranging from porencephaly to hydryanencephaly of variable severity (33). In addition to the full term lambs, some dead deformed fetuses were observed. Subsequently, researchers were able to reproduce encephalopathic lesions in approximately 21% of the ovine fetuses born to pregnant ewes which were inoculated with egg-attenuated BTV vaccine between 35 and 42 days of gestation (174). Additional studies, utilizing intrafetal inoculation at different stages of the ovine gestation, indicated that the type of congenital cerebral anomaly found in a lamb depended on the age of the fetus at the time of inoculation (123). Lambs

infected at 50 to 58 days of gestation were born with hydranencephaly while those infected at 75 to 78 days were born with porencephalic cysts. Lambs infected after 100 days of gestation were born with mild focal meningoencephalitis with no pathologic sequelae except glial nodules.

Porencephaly and cerebral necrosis were also observed in 2 near-term ovine fetuses aborted in a bluetongue-enzootic flock (140). Lesions in the fetuses were similar to those observed after experimental inoculation with bluetongue vaccine virus, although they were considered to have resulted from natural infection of the ewes.

Even though BTV was isolated from several naturally occurring cases of bovine hydranencephaly in the mid 1960's (109) and was serologically incriminated as being responsible for other similar cases during that same time (97), the condition was not reproduced experimentally in calves until several years later (12). Two bovine fetuses were inoculated <u>in utero</u> with egg-attenuated BTV, 1 at 126, and the other at 138 days of gestation. The fetus which was inoculated at 126 days of gestation was aborted 136 days post inoculation (262 days of gestation) and presented with typical hydranencephaly. The second fetus was born alive 135 days post-inoculation (273 days of gestation) and had morphologic changes in its brain characteristic of porencephaly. The fact that encephalopathies could be

induced experimentally with attenuated BTV, and that similar cases were observed under field conditions, led investigators to conclude that BTV must be considered as a possible cause of abortion and neonatal deaths in cattle.

Lesions have also been regularly produced in suckling mice used in the identification and propagation of orbiviruses. With few exceptions, the primary isolation of virus has been achieved by intracranial inoculation. The symptoms exhibited by suckling mice infected with orbiviruses (including BTV) are by no means pathognomonic, but merely suggest encephalitis (169). Initially, the mice cease to suckle, leave the nest, and become scattered. This early stage of infection is usually followed by a period of hyperactivity or by alternating periods of lethargy and hyperactivity. As the disease progresses, the animal's movements become more incoordinated and spastic, followed by coma with eventual death. Other investigators reported that the pattern of susceptibility of suckling mice to infection (inoculated intracranially with mouse-brain adapted bluetongue vaccine virus) correlated well with the maturation and migration of cells within the developing telencephalon (116). They hypothesized that susceptibility of cells decreased as the cells achieved their ultimate anatomic location and became differentiated into more mature cells. As this process occurred, the cells became committed to more specialized functions which

either led to a loss of virus receptor sites, or to a loss or acquisition of some metabolic function which might ultimately render the cells resistant to infection (116). When considered together, both of the foregoing hypotheses offer a plausible explanation for the age related variation in lesions observed in fetal and neonatal animals infected with BTV. Histologic lesions observed in mice inoculated with mouse-brain adapted BTV include endothelial hypertrophy, necrotic vasculitis and perivasculitis, vascular thrombosis, and *status spongiosus* (107). Ischemia due to vasculitis appeared to increase the severity of the lesions (107).

Bluetongue virus has been recovered post-inoculation from experimentally inoculated sheep (18) and cattle (52, 96) for up to 60 and 59 days respectively, and from naturally infected sheep (56) and cattle (72) for up to 120 and 81 days respectively, after the first clinical signs were observed. Serologic evidence has indicated that BTV infection occurs in many species of wild ruminants including elk, antelope, bighorn sheep, barbary sheep, moose and several species of deer (11, 159). Several reports (93, 97-101) have indicated that immunologic tolerance to BTV may develop in fetuses infected while *in utero*, and that these infections might result in the birth of calves that remain infected for life. Mature bulls originally infected *in utero* have been shown to be capable

of excreting the virus in semen (24), thereby providing a mechanism of perpetuating infection by vertical transmission. A unique feature of these studies is that all the viral transmissions were carried out by utilizing infected vectors (Culcicoides variipennis). It was also demonstrated that latently infected tolerant cattle became viremic several hours after exposure to non-BTV-infected vectors (37). This observation suggests that there is some type of vector-induced activation of BTV in latently infected cattle.

Due to marked variations in the severity of the symptoms and lesions, a definite diagnosis based on clinical features alone generally cannot be made. Serological methods and virus isolation procedures for the detection of BTV infection must, therefore, be relied upon to definitively detect BTV-infected animals. Currently, the MCF (16) and the AGP (84) tests are the standard serotests used for identification of BTV infection. However, results of a recent study (124) indicate that the sensitivity of AGP tests is inadequate for this purpose. AGP tests were negative for antibody in 43% of the cattle and 23% of the sheep from which BTV was isolated. New assays including a hemolysis-in-gel assay (85), radioimmunoassay, and an enzyme immunosorbent assay (78) are currently being developed. Each of these assays is designed to detect much lower levels of BTV antibody in

animal sera. The assays are also more specific than either the MCF or AGP methods (78, 85). The increased sensitivity is a necessity if an extensive control program is to be implemented.

An additional feature of BTV infection is that multiple serotypes of virus can often be isolated from infected flocks or herds (10, 124, 125). In one study (124), one third of all the ranches from which BTV isolations were made had BTV infections involving 2 or 3 serotypes. This further emphasizes the complexity of the disease and raises the possibility that infection of a susceptible animal with multiple BTV serotypes could result in formation of viral recombinants, since the BTV genome is composed of 10 segments of double-stranded RNA. Such viral recombinants could be antigenically different and, therefore, not neutralized by the host's immune response. Under such conditions, the severity of virus induced lesions could be different. This finding also points to the possible danger in using live multivalent attenuated BTV vaccines which could revert to virulence and cause abortion in pregnant females, and possibly persistence of vaccine virus in cattle (82, 124).

Although the immune response comprises only a part of host defence mechanisms, it plays an important role in limiting viral (1) as well as other infectious diseases. The host's immune response to an infectious agent usually

depends on the interation of the two arms of the immune system, i.e., cell mediated (CMI) and humoral mediated immunity (HMI). Cell mediated immunity is represented mainly by thymus lymphocytes (T cells) that act locally by contact on a 1-for-1 cell basis in solid tissue. Humoral mediated immunity is composed mainly of bursal-equivalent lymphocytes (B cells) and their products (antibodies). T Cell responses are induced relatively quickly, operating as soon as 3 to 4 days after initiation of the infection, but decline rapidly within 5 to 10 days of agent elimination (14). In contrast to T cells, antibodies usually become measurable later in a virus infection and remain at a high level for a much longer time.

Antibody responses cannot be discussed independently of T cells, since it has been demonstrated that initiation of antibody responses to most antigens is a function of the interactions between macrophages, B cells and/or T cells (31). In addition, it has been shown in recent studies that subclasses of T cells (i.e., T-helper and T-suppressor cells) when activated, directly influence the production of antibody following antigenic stimulation and that there is simultaneous coexistence of T-helper and T-suppressor cells in populations of antigen primed murine spleen cells (26). It has been subsequently demonstrated, using E. coli B-galactosidase as antigen, that sequential waves of help and suppression from T cells occur during the primary

immunologic response to specific antigenic stimulation, and that these sequential waves were in the pattern of "early help" succeeding a transient period of suppression after which the overall response was helpful (143). An early suppressive phase appeared soon after a secondary exposure to the antigen (143).

Such a regulatory scheme, should it hold true for the antibody response to BTV, may add to the understanding of the yet undefined mechanisms of disease expression in BTV infections. For example, should an animal, previously infected with BTV, be responding immunologically according to the above regulatory scheme, and simultaneously become reinfected by the same or a different serotype of BTV, then the ultimate disease expression could possibly depend upon which mode, be in help or suppression, the regulatory system happened to be in at the time of the second infection. Should the regulatory system be in the "help mode," enhancement of the antibody response would be expected and conversely, should it be in the "suppression mode," suppression of the antibody response would be expected.

Modulation of the immune and disease response by sequential or simultaneous infection by different serotypes of a virus has been observed in other viral infections. Dengue hemorrhagic fever (DHF), a syndrome observed primarily in children and characterized by acute vascular

permeability and activation of the coagulation and complement systems, is 1 example. A benign form of the disease, "classical dengue fever," occurs in single dengue virus (DV) infections of previously unexposed persons while the more severe, often fatal DHF, occurs almost exclusively in children possessing pre-infection DV antibody, whether actively or passively acquired (60). Although there are 4 types of DV recognized in nature, clinical-virological studies have shown that dengue 2 virus (DV2) is the virus most frequently isolated from DHF patients (60). Although a positive correlation has been established between sequential DV infections and the initiation of DHF not all sequential DV infections result in DHF. If the secondary exposure occurs after an extended period (5 years or more), or the sequential infection does not end with DV2, then such an infection does not initiate DHF (60). Also, if 2 or more dengue viruses (including DV2) are endemic in a given area, then sequential infections in that area do not result in DHF (60).

Feline infectious peritonitis (FIP) is another example of such a disease process. The disease affects domestic and exotic cats and is a contagious and highly fatal coronavirus (FIPV) infection (68). Natural infections with FIPV are often inapparent, although antibodies are produced in response to the infection. Only a small number of infected cats develop the fatal systemic disease and only

after several weeks to years following the initial infection (129, 130, 171). Recent experimental evidence has demonstrated an enhancement of disease in FIPV challenged kittens possessing pre-challenge FIPV antibodies, whether naturally acquired or passively administered (131). Other studies showed that clinical signs and death were seen in non-FIPV-sensitized kittens inoculated with FIPV only after an active antibody response was demonstrated (172).

In both DHF and FIP, the production or acquisition of a non-neutralizing, but cross-reacting antibody is a key feature of the disease process. In both syndromes, the respective virus has been shown to replicate and persist in mononuclear phagocytes in the presence of such non-neutralizing antibody (59, 142). Mechanistically, it has been proposed for both DHF and FIP that these non-neutralizing, but cross-reacting antibodies may promote acute disease by enhancement of virus infection in mononuclear phagocytes (opsonic activity) or by formation of immune complexes, activation of complement and secondary vascular disturbances (60, 172).

Immunity studies in sheep with BTV have demonstrated that 61.5% of the animals having low neutralizing antibody titers to 1 of 2 BTV isolates developed clinical disease when challenged with 1 of the isolates (19). Another study showed that clinical signs and lesions in sheep

experimentally inoculated with BTV occurred several days after maximum virus infection of cells, and coincided with the detection of significant levels of BTV antibody (148). These observations tend to suggest that immunopathologic processes may determine whether BTV infection in ruminants results in an inapparent process or in clinical disease, such as occurs in DHF and experimental FIP.

CHAPTER III

APPLICATION OF AVIDIN/BIOTIN-ENHANCED ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF BLUETONGUE VIRUS ANTIBODIES

Introduction

Bluetongue virus (BTV) is an arthropod-borne agent known to infect many ruminant species (20, 69, 70, 158). Bluetongue, the clinically recognized disease, is observed only as limited outbreaks in certain herds or flocks (20), although BTV infection is widespread in nature (11, 19, 124). The principal concern of bluetongue-free countries is the inadvertent importation of BTV-infected livestock. Recent reports of BTV isolation, and the demonstration of anti-BTV titers in animals from countries previously considered to be BTV-free, justify the concern of these countries (78, 151). Cattle and sheep may harbor infectious BTV for long periods without overt clinical disease (48, 97). When BTV infection results in clinical illness, the extreme variability of disease expression often precludes a specific diagnosis based solely on clinical signs (20, 69, 70, 158).

Currently, the modified complement fixation (MCF) (16) and agar gel precipitant (AGP) (84) tests are the standard serological tests used by concerned countries as a determinant of BTV infection. These assays, however, leave

much to be desired as routine diagnostic aids in the identification of bluetongue virus infected animals. The many variables involved in the MCF tests often create technical problems with the assay as do the anticomplementary properties of some bovine and ovine sera (71). The AGP test is relatively free of technical problems, and is easily adapted to large-scale screening programs (85, 88, 110). However, there is some concern as to the sensitivity of the assay. It was demonstrated in a recent study that 43% of the BTV isolated from cattle, and 23% from sheep, came from AGP tested seronegative animals (124).

It is evident that more sensitive and reproducible assays will be required for any type of effective BTV control program. One such assay, the enzyme-linked immunosorbent assay (ELISA), has rapidly gained wide application in the biomedical field. The assay was first introduced in 1971 as an alternative to the highly sensitive radioimmunoassay (42, 163). It is a practical, relatively simple, inexpensive, and extremely sensitive procedure (42, 43, 78, 163). Compared with tests routinely used for serodiagnosis, the ELISA generally has been found to be more sensitive (43, 78, 87, 128, 137, 162). Also, the assay has a wide range of applicability, and has been adapted for use with many infectious agents including BTV (43, 78, 87, 128, 162).

Before the ELISA can be adapted for use with different antigens, several variables need to be examined to achieve the maximum potential of the assay. The coating buffer used to adsorb antigen to the solid phase, is critical to the assay. Several buffers, as well as different pH's of the same buffer, have used as coating buffers with various antigens (17, 87, 128, 139, 160, 162, 166). The preparation and concentration of antigen for the ELISA is also critical in achieving optimal results. Some assays require highly purified antigen, while others have utilized crude suspensions of lysed cells or tissues. In addition, some antigens apparently adhere better to 1 type of plastic than to another and; therefore, it is advantageous to evaluate several different microtiter plates for use with the antigen to be studied.

Nonspecific binding of serum proteins or enzyme conjugate to the microtiter plate has been a major problem with some ELISA procedures. This type of binding can reduce the sensitivity and specificity of the assay by resulting in unwanted background staining. The addition of various protein solutions and/or nonionic detergents to the reagents dilutions, and to plate rinsing solutions, often will reduce the amount of background staining resulting from binding of nonspecific protein.

Recently, an improvement was made to the ELISA technique when the newly developed avidin/biotin-peroxidase

complex (ABC) methodology was adapted to the ELISA from an immunoenzymatic tissue staining procedure. The ABC staining procedure makes use of the extraordinarily high binding affinity of avidin for biotin (dissociation constant of 10^{-15}M). Avidin is a 68,000 molecular weight glycoprotein present in egg white which has 4 binding sites for biotin, a small molecular weight vitamin (73). The ABC (Fig. 1) is produced by incubating excess avidin with biotin conjugated to peroxidase enzyme. Avidin serves as a link between multiple biotin-labeled peroxidase molecules. Each peroxidase enzyme molecule is conjugated with several biotin molecules, therefore, functioning as a bridge between avidin molecules. Consequently, a "lattice" is formed which contains several peroxidase enzyme molecules for each molecule of the original immunoglobulin (primary antibody) being quantitated. The potential for high staining intensity and reduced background staining is achieved when the complex is bound to the solid phase by means of the biotin-labled secondary antibody (73). The ABC system is available as pre-titrated kits (Vectastain ABC Kit, Vector Laboratories, Inc., Burlingame, CA) which greatly simplifies the assay procedure.

An avidin/biotin-enhanced ELISA (A/B-ELISA) has not yet been applied to the detection of BTV antibodies. The present study was undertaken to determine if the A/B-ELISA could be adapted for use with BTV in a mouse model system,

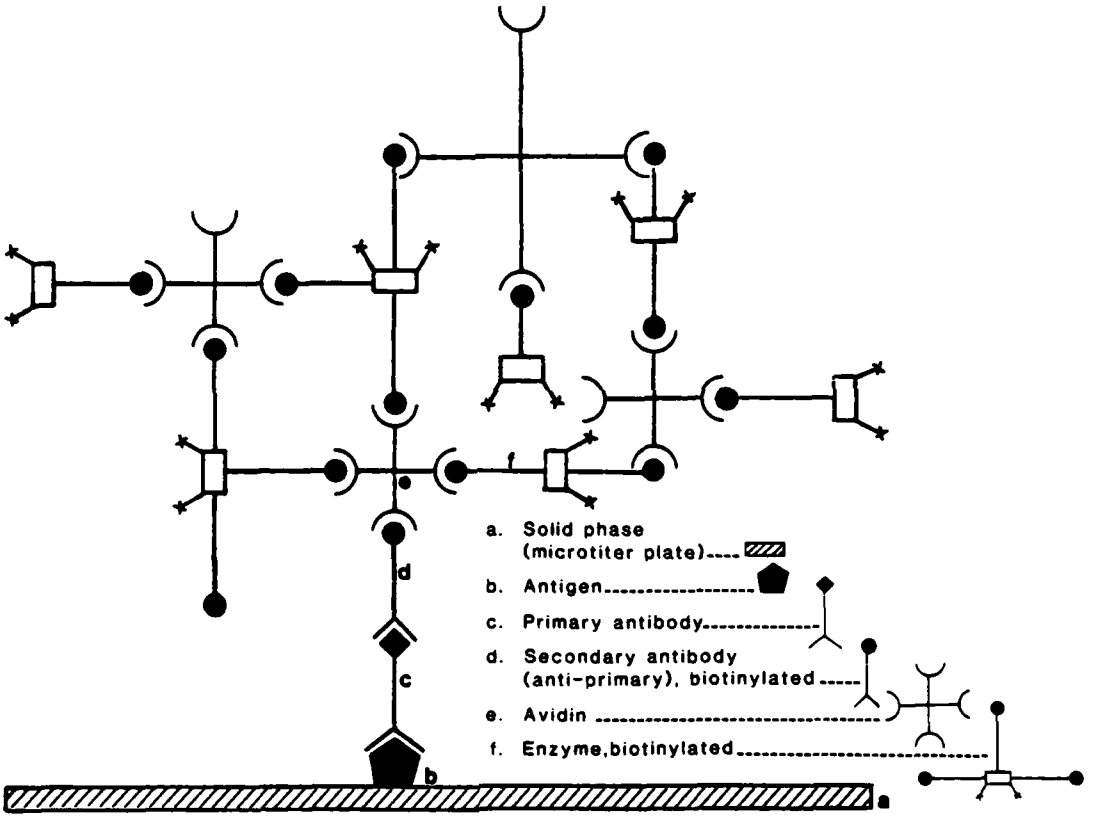

Fig. 1. Schematic representation of the avidin/biotin-peroxidase complex (ABC).

and to determine the conditions necessary to obtain optimal test results with the assay. Nonetheless, the assay is ideally suited for screening procedures, and it is possible that the ultrasensitive nature of the ABC system might facilitate the identification of BTV and aid in the development of effective control programs.

Materials and Methods

Virus Source and Propagation

The 4 American serotypes of BTV [low passage isolates in Oxylated-Carbol-Glycerin solution (OCG)-treated sheep blood] were obtained from Dr. Stewart McConnell (Department of Veterinary Microbiology and Parasitology, Texas A & M University, College Station, Tx.), and initially grown in Vero cells (African green monkey kidney cells), less than 10 passages for all serotypes, and subsequently adapted to mice. Newborn (1- to 3-day old) white Swiss mice (parent stock supplied by Charles River Breeding Laboratory, Inc., Washington, MA) were inoculated intracerebrally with 0.02 ml of harvested tissue culture virus. Infected suckling mice were euthanized 3 to 6 days post-inoculation and the brains were asceptically removed and a 10% brain suspension was prepared in Dulbecco's minimum essential media (DMEM). The brain suspension, labeled P_1, served as the inoculum for a second litter of newborn mice. The procedure was repeated until a fifth passage brain suspension (P_5) was

obtained. The stock P_5 mouse brain suspensions of each individual serotype were aliquoted and stored at -70°C. The P_5 stock aliquotes were titrated at dilutions from 10^{-1} through 10^{-5}. Groups of 6 neonatal C57Bl/6 mice (C57, parental stock obtained from the Jackson Laboratory, Bar Harbor, ME) were inoculated intracerebrally with 0.02 ml of each dilution and housed with their dams. Four days after the last recorded signs of illness or death, varying from a minimum of 10 days with BTV serotypes 11 and 17 to a maximum of 21 days with serotype 13, the experiment was terminated; and the number of mice which had died and those which had survived were tabulated for each serotype dilution. There were no trauma-induced deaths during the first 24 hours. All subsequent deaths were interpretated as being virus-induced. The minimum lethal dose for 50% of the neonatal mice (MLD_{50}) was calculated for each of the 4 BTV serotypes using the Reed-Muensch method (133) and the infectivity levels, presented in Table 1, were denoted as the MLD_{50}/ml of original 10% brain suspension.

Preparation of Antigen

Two methods for the production of BTV antigen were utilized, and the resulting antigens compared during the application of the A/B-ELISA. The first method (88) utilized BTV infected cell cultures as the source of viral antigen. Briefly, Vero cells were grown to a confluent

TABLE 1. CALCULATED MINIMUM LETHAL DOSE INFECTIVITY LEVELS (MLD_{50}) OF THE STOCK FIFTH PASSAGE (P_5) MOUSE BRAIN SUSPENSIONS OF BLUETONGUE VIRUS (BTV)

BTV Serotype	MLD50/ml
BTV 10	1.58×10^4
BTV 11	8.89×10^4
BTV 13	2.82×10^3
BTV 17	2.81×10^5

monolayer in 1/2 gallon plastic roller bottle rotated approximately 1 rotation per minute (RPM) at 37°C. The culture was infected by pouring off the maintenance media, adding 10 ml of Vero-adapted BTV and rotating the bottle for 1 hour at 37°C. Following the adsorption period, the viral inoculum was decanted. Fifty ml of maintenance media (without serum) were added to the culture, and the bottle returned to the roller apparatus. When the viral cytopathic effect (CPE) reached 100% (approximately 5 days), 25 ml of 3% nonionic detergent, sorbitan mono-oleate ployoxyethylene (Tween-80) in phosphate buffered saline (PBS), were added to solubilize the cell membranes; and the culture rotated for an additional 2 hours at 3 RPM at 37°C. The fluid and cell debris were decanted and centrifuged at 2,000 X g for 15 minutes at 4°C. The supernatant fluid was then transferred to a flask and the antigen precipitated by slowly adding 1/2 the supernatant volume of a cold, saturated solution of ammonium sulfate while stirring. The sediment was discarded, and the remainder of the suspension was transferred to straight-walled centrifuge tubes and allowed to stand at 4°C for 30 minutes. The mixture was then centrifuged at 3,000 X g for 30 minutes at 4°C. The thin layer of yellow, semi-solid material (antigen "butter") which formed at the top of the mixture was transferred to a conical centrifuge tube, using a small stainless steel spatula, and centrifuged at 2,000 X g for

10 minutes at 4°C. The clear salt solution found under the layer of antigen "butter" was aspirated and discarded before the "butter" was dissolved in an equal volume of buffered saline [i.e., cyclohexylamino-ethanesulfonic acid (CHES), Sigma Chemical Company, St. Louis, MO.]. When resuspension was complete, the antigen solution was further diluted with 1 volume of PBS. Sodium azide was added to a final concentration of 0.02%, and the resulting stock antigen solution aliquoted and stored at 4°C.

The second method of BTV antigen production involved the fluorocarbon extraction of antigen from infected neonatal mouse brains (53). Briefly, 1- to 3-day old C57 mice were inoculated intracerebrally with 0.02 ml each of undiluted P_5/BTV mouse brain suspension. The litters were housed with their dams. Brains of the inoculated mice were harvested at 3 to 6 days post-inoculation when the mice were moribund. Following harvest, a 20% suspension of homogenized brain in 8.5% sucrose was added to an equal volume of fluorocarbon, trichlorotrifluoroethane (Freon 113, Eastman Kodak, Rochester, NY), mixed in an electric homogenizer (Virtis Research Equipment Co., Gardiner, NY) and centrufuged at 6,500 X g for 30 minutes at 4°C. The aqueous phase was collected and residual Freon 113 was removed under vacuum. The resulting stock antigen solution was aliquoted and stored at -70°C. Antigen was prepared from BTV 11, BTV 13, and BTV 17 for use throughout the

present study.

Preparation of Antisera

Thirty adult C57 mice were divided into 3 groups of 10 mice each. Each mouse in one group was inoculated intraperitoneally (IP) with 0.5ml (4.45×10^4 MLD_{50}) of thawed undiluted P_5/BTV 11 mouse brain suspension once a week for a total of 3 inoculations. Mice in the second and third groups were inoculated in a similar manner with P_5/BTV 13 (1.41×10^3 MLD_{50}) and P_5/BTV 17 (1.41×10^5 MLD_{50}) mouse brain suspensions, respectively.

Two weeks following the third inoculations, all mice were exsanguinated and their serum pooled as per individual groups, aliquoted, and stored at -70°C as stock anti-BTV sera. Normal control serum obtained from nonimmunized C57 mice was aliquoted and stored at -70°C as stock normal serum.

Vectastain ABC Kit

Biotinylated, affinity-purified horse anti-mouse immunoglobulin G (IgG), affinity-purified avidin (Reagent A), and biotinylated horseradish peroxidase (Reagent B) solution were obtained in dropper-bottle kits (Vector Laboratories, Inc., Burlingame, CA). The working dilution of biotinylated antibody was prepared by the addition of 1 drop (approximately 45 ul) of the stock solution to 10 ml

of buffer. The ABC reagent (avidin/biotin-peroxidase complex) was prepared by adding 2 drops (approximately 90 ul) of Reagent A to 10 ml of buffer and subsequently adding 2 drops (approximately 90 ul) of Reagent B to the same container of buffer. The ABC solution was mixed and allowed to stand at room temperature (RT) for 5 minutes before use.

Substrate Solution

The substrate for peroxidase activity consisted of a solution of ortho-phenylenediamine dihydrochloride (OPD), Sigma Chemical Co., St. Louis, MO) and hydrogen peroxide. The substrate was made prior to use by mixing equal parts of 0.3% hydrogen peroxide (freshly prepared from a concentrated stock), and a 0.08% OPD solution (0.8 mg/ml in 0.1M citrate buffer, pH 5.0). Once prepared, the solution was kept in the dark at 4°C. Sulfuric acid (4N H_2SO_4) was used to stop the enzyme-substrate reaction.

Coating Buffer

Several coating buffer preparations, some from previously reported ELISA procedures (17, 87, 129, 139, 160, 162, 165), were used in the adaption of A/B-ELISA for use with BTV. The buffers evaluted were:

1. 0.05M carbonate buffer, pH 9.0
2. 0.05M carbonate buffer, pH 9.6
3. 0.05M PBS, pH 7.6

4. 0.05M PBS, pH 8.0
5. 0.05M PBS, pH 8.5
6. 0.06M acetate buffer, pH 8.2
7. 0.05M glycine buffer, pH 10.0

Bluetongue virus antigen was diluted 1:200 in the respective buffers and delivered in 50 ul amounts into wells of microtiter plates. The plates were covered and stored overnight (12 to 18 hours) at 4°C. Although antigen coated plates remained usable when stored at 4°C for several days, better results were obtained by preparing the plates 12 to 18 hours before expected use.

Diluent Solution

Several solutions were tested for use as diluents of the ABC reagent and the test sera in an effort to minimize nonspecific background staining in the test wells. Those solutions tested were:

1. 0.01M PBS, pH 7.6, containing 0.05% sorbitan monolaurate polyoxyethylene (Tween 20)
2. 0.01M PBS, pH 7.6, containing 0.05% Tween 20 and 0.05% crystalline grade bovine serum albumin (BSA)
3. 0.01M PBS, pH 7.6, containing 0.05% Tween 20 and 1.0% BSA
4. 0.01M PBS, pH 7.6, containing 0.01% Tween 20 1.0% BSA, and 0.05% gelatin--(PTBG)
5. 0.05M Tris (hydroxymethyl) aminomethane buffer (Tris), pH 7.6
6. 0.05M Tris, pH 7.6, containing 5% normal horse serum (NHS)
7. 0.05M Tris, pH 7.6, containing 5% fetal calf serum (FCS)
8. 0.05M Tris, pH 7.6, containing 5% tryptose phosphate broth (TTB)

All sera used in the assay, including the horse anti-mouse IgG, were diluted in each of the above solutions during the diluent experiments.

Microtiter Plate Washer and Rinse Solution

Zero point 15M (0.15M) sodium chloride solution containing 0.05% Tween 80 was used as the rinse solution for microtiter plates. The solution was used with a semi-automatic microtiter plate washer (Dynawasher II, Dynatech Laboratories, Inc., Santa Monica, CA).

Absorbancy Readings

Absorbancy readings were recorded at 490 nm with an automatic multichannel spectrophotometer with digital readout and instant print-out mechanism (MR 580 Microelisa Auto Reader, Dynatech Laboratories, Inc.).

Microtiter Plate

The Immulon II flat bottom microtitration plate (Dynatech Laboratories, Inc.) was recommended for use with the plate washer and plate reader utilized in the present study. The plate was designed specifically for the ELISA procedure, and because favorable results were obtained with this plate, its use was continued throughout the study. Other plates examined and found to be less desirable included: micro hemagglutination plates (Cooke Microtiter

M29AR) and flat and round bottom microtiter plates (Micro ELISA plates, Dynatech Laboratories, Inc.).

General A/B-ELISA Procedure

The assay procedure (Fig. 2) was a modification of that supplied with the ABC staining kit. Briefly, BTV antigen was diluted 1:200 in coating buffer, and 50 ul amounts were delivered to wells of a microtiter plate. The plate was covered and stored at 4°C overnight. At the time the assay was to be performed, the plate was washed and 1:100 dilutions of anti-BTV, and normal serum, were delivered to appropriate wells on the plate in 50 ul amounts. The plate was incubated 1 hour at room temperature (RT) and rinsed. Working strength biotinylated horse anti-mouse IgG (from the kit) was added to each well in 50 ul amounts. The plate was incubated 30 minutes at RT and rinsed again before working strength ABC reagent was added to each well in 50 ul amounts. A 15 minute RT incubation followed, after which the plate was again rinsed before 100 ul of substrate was added to each well. The enzymatic reaction was stopped following a 30 minute RT incubation in the dark, by the addition of 100 ul of 4N H_2SO_4 to each well. The absorbance of each well was determined at 490 nm.

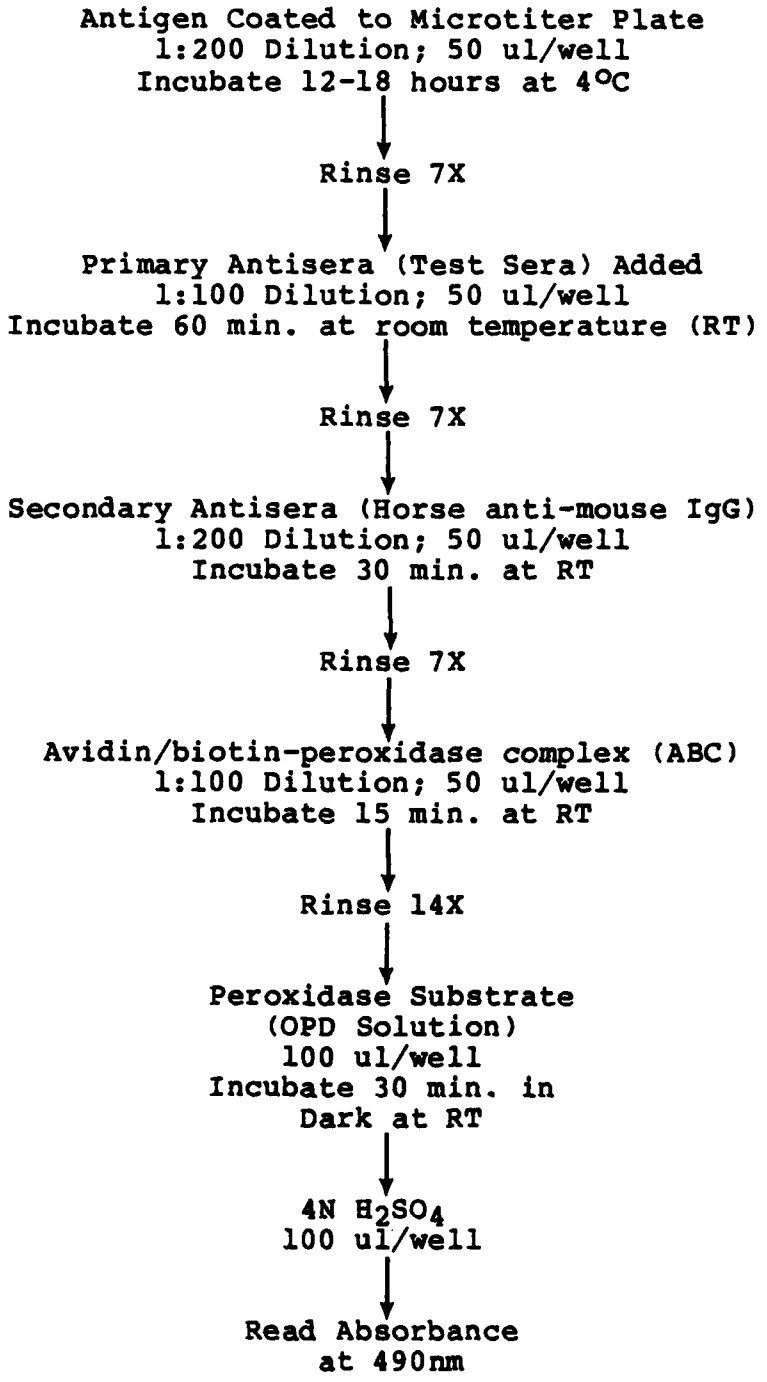

Fig. 2. General procedure for the avidin/biotin-enhanced enzyme-linked immunosorbent assay (A/B-ELISA).

Results

Determination of Optimal Antigen and Serum Dilutions

Twofold serial dilutions of tissue culture and mouse brain BTV 11 antigens from 1:50 through 1:400 were titrated against twofold serial dilutions of stock anti-BTV 11 serum from 1:50 through 1:800 in a standard checkerboard procedure utilizing the A/B-ELISA. Carbonate buffer, pH 9.6, and Tris buffer, pH 7.6, were used as the coating buffer and reagent diluent, respectively, as recommended by the ABC staining procedure. Briefly, the procedure was as follows. Stock BTV 11 antigens were diluted 1:50, 1:100, 1:200 and 1:400 in carbonate buffer. Fifty microliter amounts of each dilution were delivered to 12 wells (horizontally) of a microtiter plate, and the plate was stored at 4^{o}C overnight. At the time of assay, the plate was washed 7 times to remove excess unbound antigen. Stock anti-BTV 11, and normal mouse serum were diluted 1:50, 1:100, 1:200, 1:400 and 1:800 in Tris buffer, and each delivered in 50 ul amounts to a vertical row of wells on the plate. Two columns of blank wells (no serum) were included for each antigen row. The remainder of the procedure was carried out as described in the materials and methods for the A/B-ELISA procedure. Results of this checkerboard titration (Table 2) indicated a lack of obvious correlation between the titration scheme and the

TABLE 2. INITIAL COMPARISON[a] OF MOUSE BRAIN AND TISSUE CULTURE SOURCE OF BLUETONGUE VIRUS SEROTYPE 11 (BTV 11) ANTIGEN BY TITRATION OF POSITIVE AND NEGATIVE SERUM.

Antigen type and dilution	Dilution of Positive Serum						Dilution of Negative Serum					
	50	100	200	400	800	Blank	50	100	200	400	800	Blank
Mouse Brain BTV 11												
50	.780	.705	.740	.650	.433	.110	.439	.446	.374	.280	.239	.138
100	.818	.752	.686	.622	.482	.119	.501	.362	.305	.295	.251	.130
200	.795	.783	.681	.615	.401	.089	.482	.330	.308	.297	.247	.105
400	.742	.692	.622	.593	.403	.100	.448	.318	.283	.276	.232	.110
Tissue Culture BTV 11												
50	.293	.833	.138	.323	.204	.134	.015	.152	.080	.031	.173	.101
100	.665	.005	.096	.065	.688	.102	.050	.251	.030	.288	.180	.089
200	.538	.033	.714	.104	.340	.100	.292	.201	.099	.101	.389	.110
400	.264	.236	.170	.134	.731	.118	.209	.114	.161	.181	.277	.109

[a]Data expressed as absorbance values at 490 nm

absorbance values of the tissue culture antigen wells. The higher absorbance values indicating specific antibody reaction with BTV antigen were haphazardly arrayed and uninterpretable. In contrast, the absorbance values of the mouse brain antigen wells correlated well with antibody and antigen dilution. To facilitate comparison of the serum and antigen dilutions or other variables on the difference between positive and negative serum absorbance values, the results were converted to a ratio value. This ratio value was obtained by dividing the absorbance values of the various positive or test serum dilutions by the absorbance values of the corresponding dilutions of normal mouse serum. The resulting calculated values were designated binding ratios. By this method, the larger the binding ratio for a test sample, the greater the separation between known positive and negative sera under the defined conditions. The largest binding ratio was demonstrated for the well corresponding to a 1:200 dilution of antigen and a 1:100 dilution of anti-serum (Fig. 3). This combination of antigen and anti-serum dilution was utilized throughout the remainder of the study.

Determination of Optimal Antigen Coating Buffer

Once the optimal dilutions of antigen and anti-serum was determined, several coating buffers were evaluated to select the best buffer for use with the BTV system. The

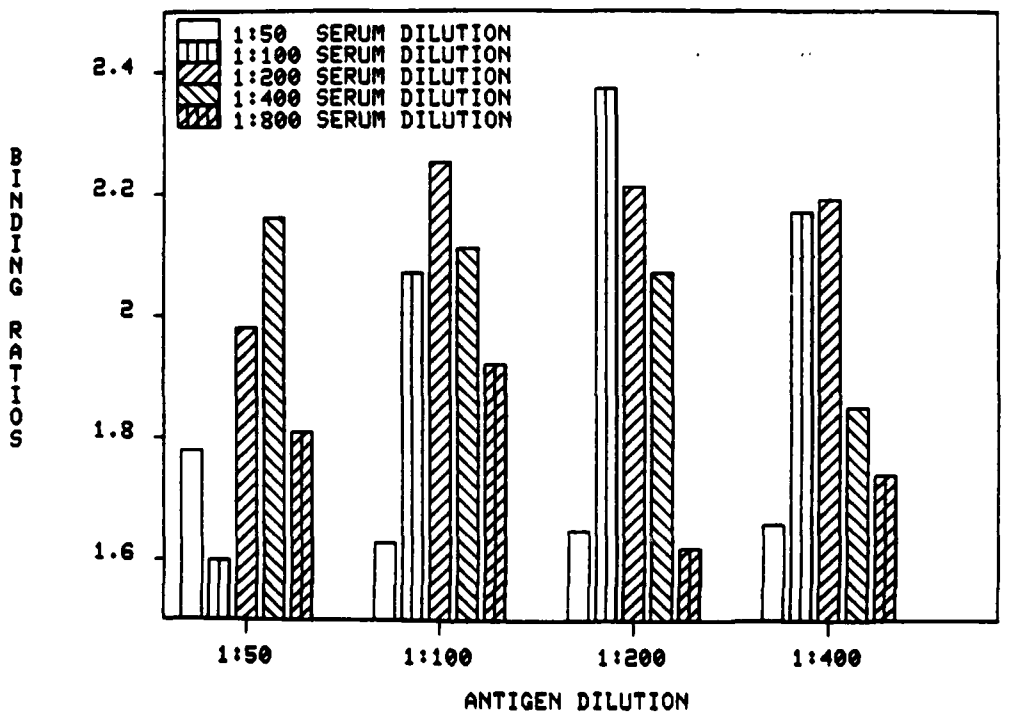

Fig 3. Binding ratios calculated for varying dilutions of bluetongue virus serotype 11 (BTV 11) antigen and antiserum.

assay procedure was basically that described in the materials and methods section above. Various coating buffers were used to adsorb the BTV antigen to the microtiter plate. Tris buffer, pH 7.6, was retained as the reagent diluent. The results of these experiments were summarized as binding ratios (Fig. 4, 5 and 6). The overall trend of the data indicated that the better results were obtained with the 1:200 dilution of antigen in most of the buffers tested, glycine being the lone exception. The uncharacteristically high value calculated for the 1:800 dilution of antigen in acetate buffer was attributed to mechanical error during the plate rinsing procedures.

The results indicated that the carbonate buffer at pH 9.6 not only demonstrated the highest calculated value at the 1:200 dilution, but it also gave the more consistent calculated values for the 3 lower antigen dilutions. Based on these results, the pH 9.6 carbonate buffer was selected and used as the coating buffer in all subsequent experiments.

Effect of Various Reagent Diluents on the Sensitivity of A/B-ELISA

In an effort to decrease the amount of background staining which might be due to nonspecific binding to the microtiter plate, several solutions were evaluated as serum diluents in the assay procedure. The assay was basically

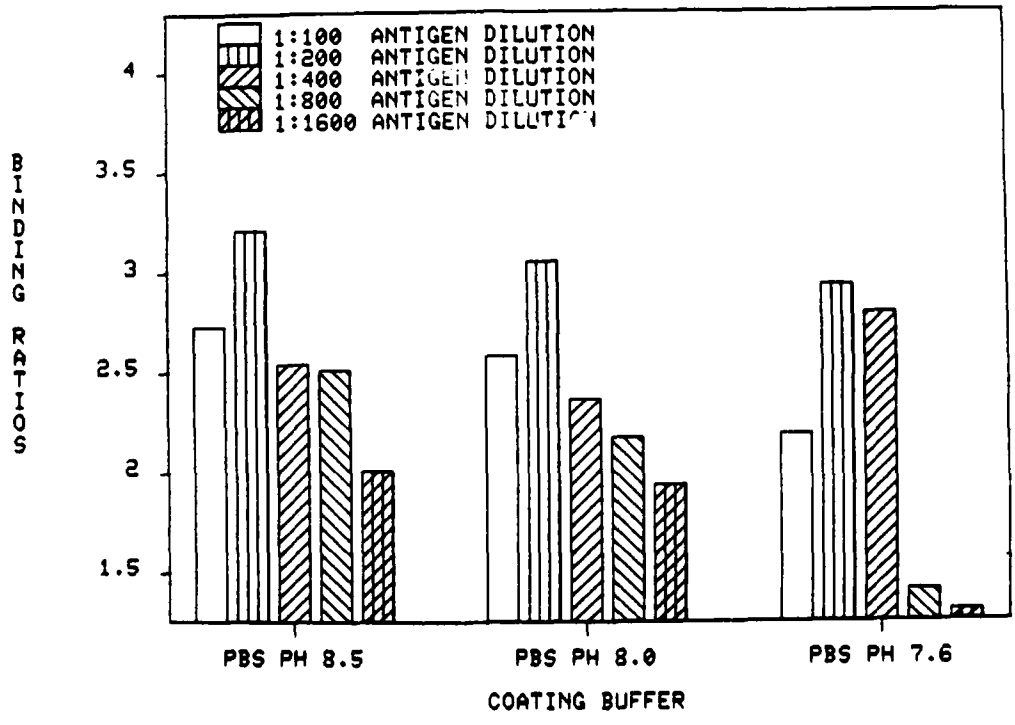

Fig. 4. Binding ratios calculated for phosphate buffered saline (PBS), pH 7.6, 8.0 and 8.5, used as coating buffer for bluetongue virus serotype 11 (BTV 11) antigen.[a]

[a]All tests conducted with 1:100 dilution of anti-BTV 11 serum.

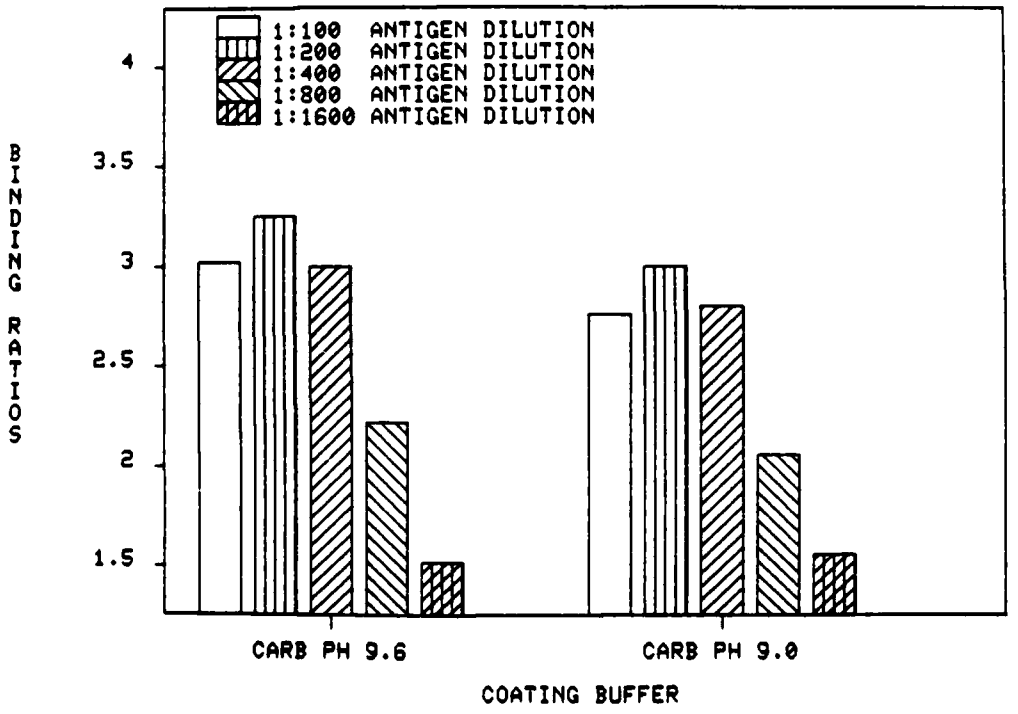

Fig 5. Binding ratios calculated for carbonate buffer, pH 9.0 and 9.6, used as coating buffer for bluetongue virus serotype 11 (BTV 11) antigen.[a]

[a]All test conducted with 1:100 dilution of anti-BTV 11 serum.

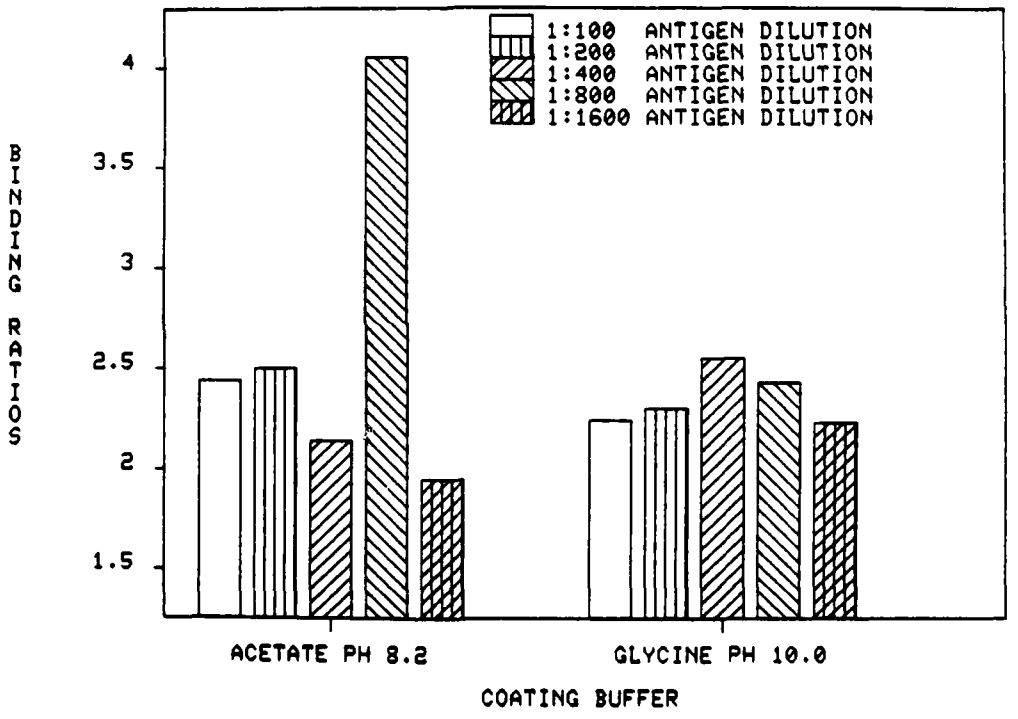

Fig. 6. Binding ratios calculated for acetate buffer, pH 8.2, and glycine buffer, pH 10.0, used as coating buffers for bluetongue virus serotype 11 (BTV 11) antigen.[a]

[a]All test conducted with 1:100 dilution of anti-BTV 11 serum.

that described in the materials and methods above. Carbonate buffer, pH 9.6 was used as coating buffer, and various solutions were evaluated as serum diluents. Results of this experiment were summarized as binding ratios (Fig. 7). The combination of PBS, Tween 20 and a nonspecific protein, i.e., BSA or BSA and gelatin, all gave better results than either Tris buffer alone, or combinations of Tris buffer and a nonspecific protein. The binding ratios for all of the PBS solutions containing a nonspecific protein were higher than the binding ratios of all the test samples in the initial titration experiment (Fig. 3, p 42) which indicated that there was greater separation between positive and negative sera and therefore, greater sensitivity. The PTBG solution, represented by the fourth graphic bar in Fig. 7, consisting of PBS, Tween 20, BSA and gelatin, was used as a diluent in a previous ELISA study (80) and, based on results of the present experiment, was selected as the serum diluent for the A/B-ELISA.

Background or nonspecific staining might also result from the nonspecific binding of enzyme conjugate to the microtiter plate and therefore, working dilutions of the ABC reagent were also made in PTBG.

The checkerboard titration procedure was repeated utilizing carbonate coating buffer, pH 9.6, and PTBG as the diluting solution for the primary and secondary antisera

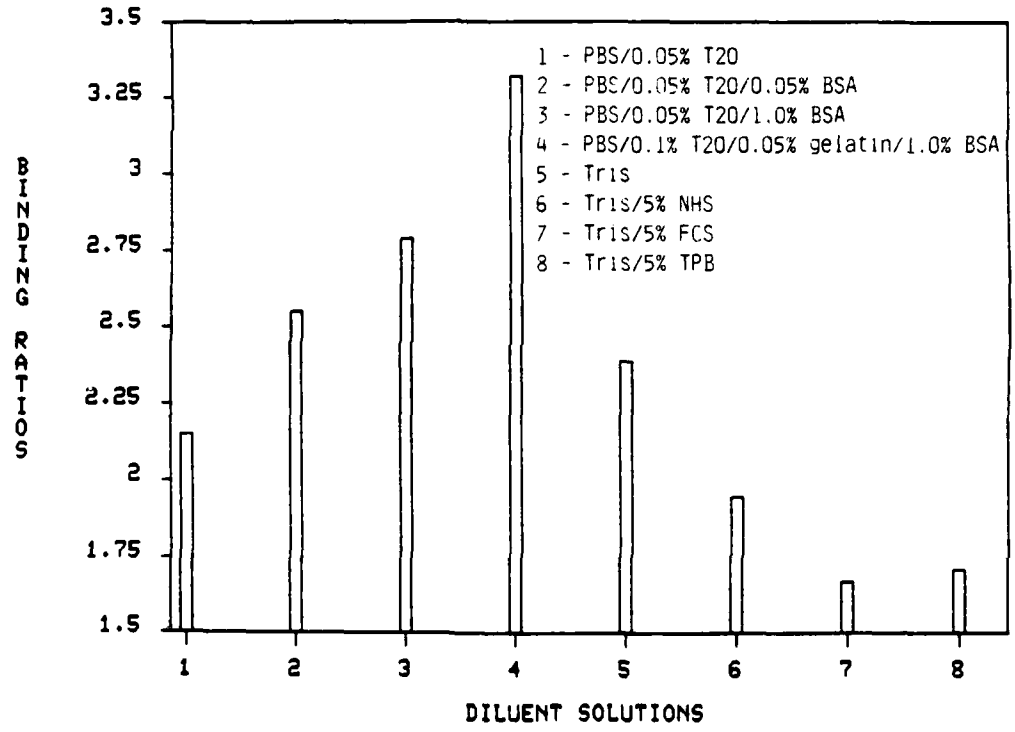

Fig 7. Binding ratios calculated for various diluents of the primary serum in the avidin/biotin-enhanced enzyme-linked immunosorbent assay (A/B-ELISA).[a]

[a]Bluetongue virus serotype 11 (BTV 11) antigen diluted 1:200 and antiserum diluted 1:100.

and the ABC reagent. Results of this experiment (Table 3) were in agreement with those of the initial titration experiment. No attempts were made to interpret data from the wells coated with tissue culture antigen as once again there was no correlation between the titration scheme and the haphazardly arrayed absorbance values. The results from wells coated with mouse brain antigen were summarized as binding ratios in Fig. 8. The results reconfirmed that a 1:200 dilution of BTV 11 antigen and a 1:100 dilution of anti-BTV 11 serum were the optimal dilutions for use in the A/B-ELISA. The relatively higher calculated values demonstrated in this experiment as compared to those of the first titration were interesting. The higher binding ratios indicated that there was greater separation between positive and negative sera when optimalbuffers were utilized.

Evaluation of Group Specificity of A/B-ELISA for BTV Antibodies

An experiment was designed to test the group specificity of the A/B-ELISA. Dilutions of BTV hyperimmune sera (anti-BTV 11, anti-BTV 13 and anti-BTV 17), normal mouse serum, and mouse anti-sheep red blood cell (SRBC) serum were assayed against 3 different BTV antigens (BTV 11, BTV 13 and BTV 17). The results of this experiment were summarized as binding ratios (Fig. 9, 10 and 11).

TABLE 3. FINAL COMPARISON[a] OF MOUSE BRAIN AND TISSUE CULTURE SOURCE OF BLUETONGUE VIRUS SEROTYPE 11 (BTV 11) ANTIGEN BY TITRATION OF POSITIVE AND NEGATIVE SERUM USING OPTIMUM COATING[b] AND DILUENT[c] BUFFERS

Antigen type and dilution	Dilution of Positive Serum						Dilution of Negative Serum					
	50	100	200	400	800	Blank	50	100	200	400	800	Blank
Mouse Brain BTV 11												
50	.525	.508	.436	.327	.285	.032	.220	.190	.168	.115	.111	.014
100	.720	.580	.473	.386	.372	.018	.365	.185	.155	.128	.143	.009
200	.818	.784	.519	.406	.390	.022	.379	.222	.172	.151	.125	.012
400	.742	.658	.561	.428	.378	.013	.347	.219	.180	.160	.135	.017
Tissue Culture BTV 11												
50	.124	.083	.149	.103	.068	.009	.159	.043	.046	.012	.026	.018
100	.569	.076	.078	.276	.068	.064	.032	.017	.024	.589	.016	.013
200	.267	.114	.098	.258	.230	.043	.043	.048	.033	.038	.026	.047
400	.030	.044	.055	.031	.057	.001	.018	.026	.035	.221	.333	.025

[a]Data expressed as absorbance values at 490 nm

[b]Carbonate buffer, pH 9.6

[c]Phosphate buffer containing gelatin, bovine serum albumin and Tween 20 (PBGT)

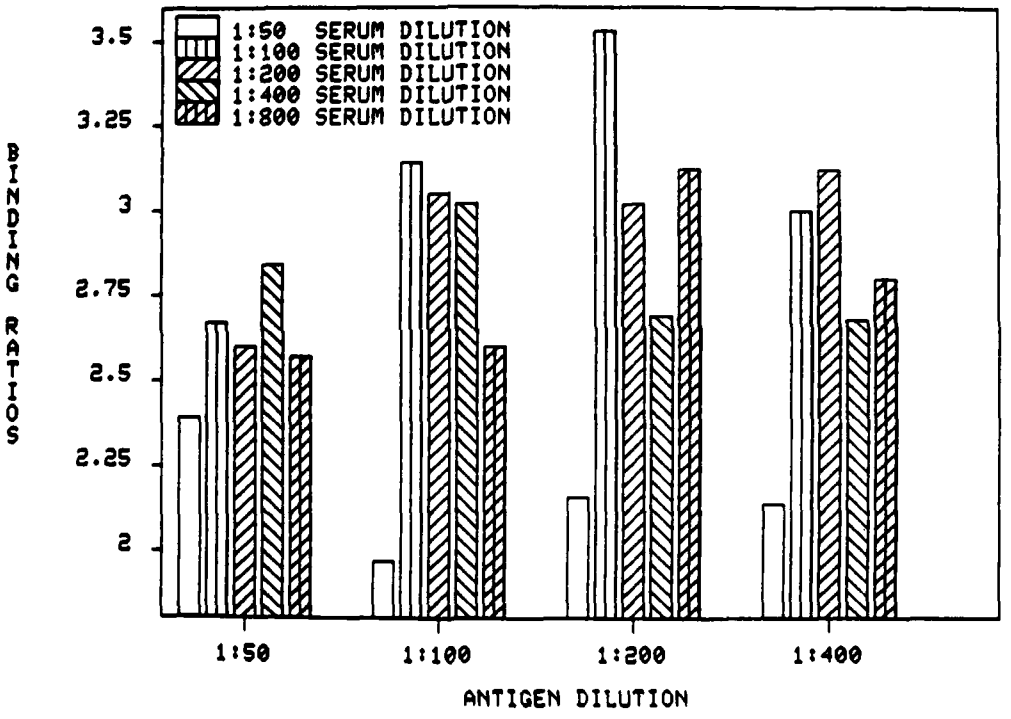

Fig. 8. Binding ratios calculated for varying dilutions of bluetongue virus serotype 11 (BTV 11) antigen and antiserum utilizing optimum coating[a] and diluent[b] buffers.

[a]carbonate buffer, pH 9.6
[b] PTBG solution

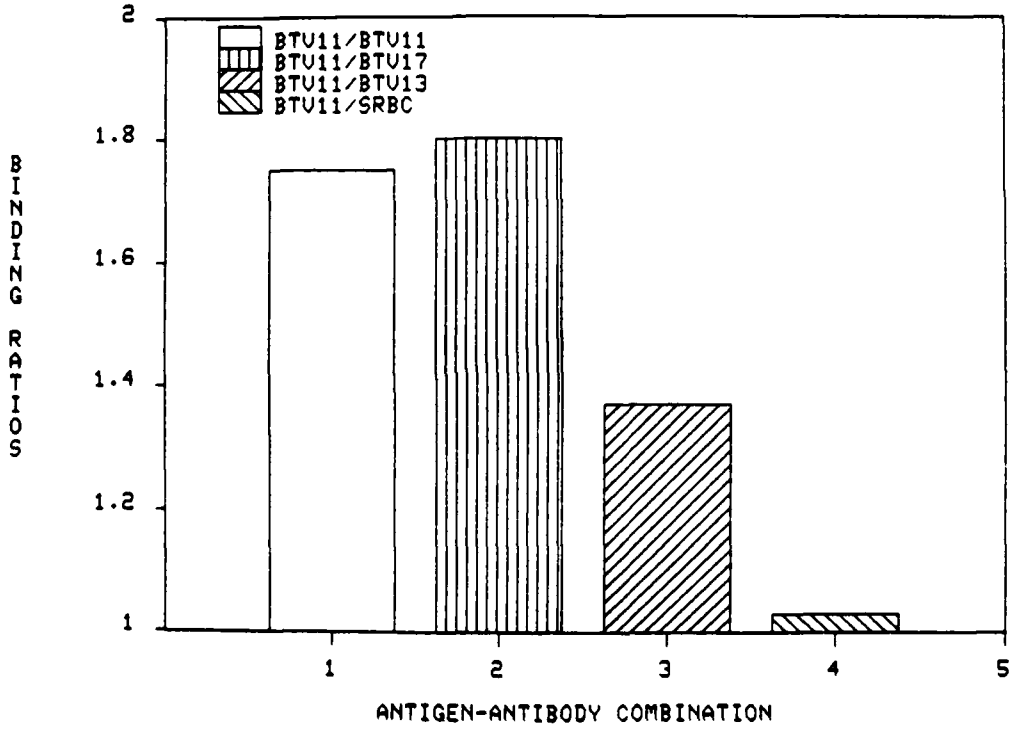

Fig. 9. Evaluation of bluetongue virus (BTV) serotype cross-reactivity in the avidin/biotin-enhanced enzyme-linked immunosorbent assay (A/B-ELISA)[a] utilizing bluetongue virus serotype 11 (BTV 11) as antigen.

[a]Absorbance value of normal serum was 0.414. Absorbance read at 490 nm.

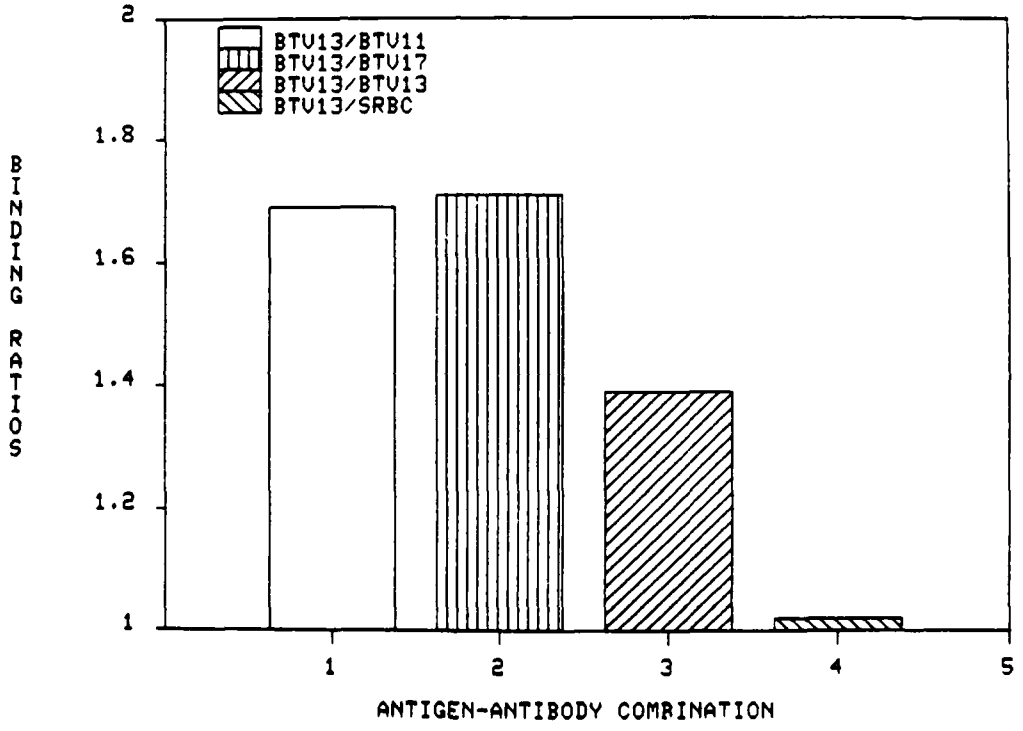

Fig 10. Evaluation of bluetongue virus (BTV) serotype cross-reactivity in the avidin/biotin-enhanced enzyme-linked immunosorbent assay (A/B-ELISA)[a] utilizing bluetongue virus serotype 13 (BTV 13) as antigen.

[a]Absorbance value of normal serum was 0.321. Absorbance read at 490nm.

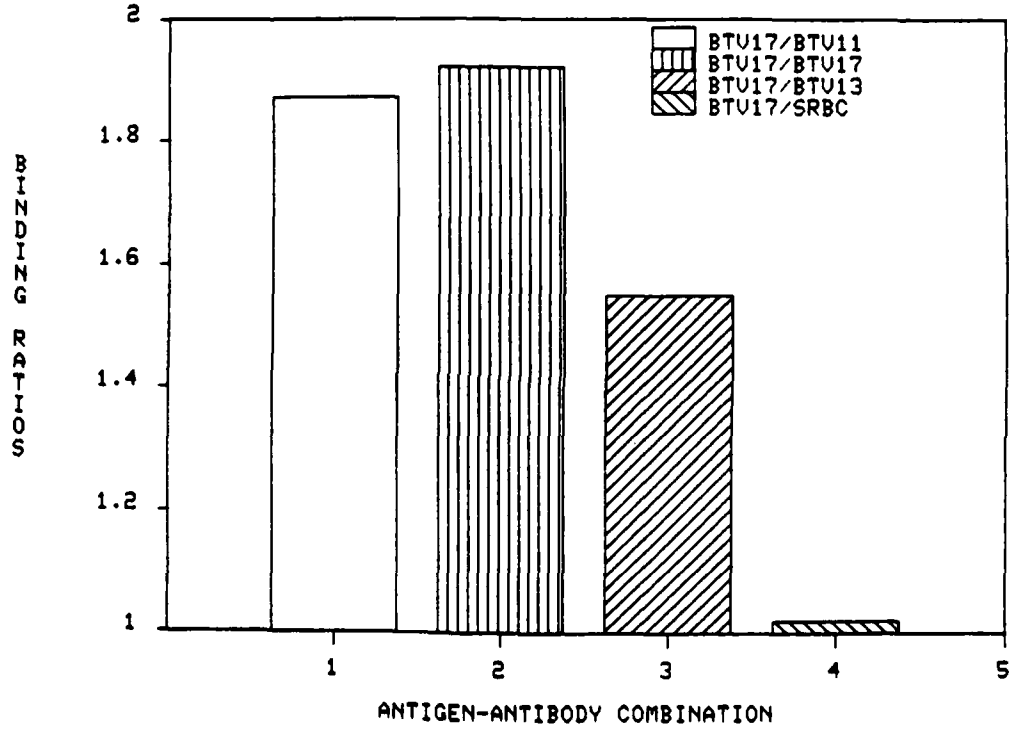

Fig. 11. Evaluation of bluetongue virus (BTV) serotype cross-reactivity in the avidin/biotin-enhanced enzyme-linked immunosorbent assay (A/B-ELISA)[a] utilizing bluetongue virus serotype 17 as antigen.

[a]Absorbance value of normal serum was 0.300. Absorbance read at 490 nm.

These data indicated that antigen not only was recognized by antibody raised against itself but also by antibody raised against 2 other serotype viruses. However, all 3 of the BTV antigens failed to be recognized by antibody raised against the unrelated antigen, SRBC. The results clearly demonstrated the group specificity of the A/B-ELISA for BTV.

Other Observations Critical to A/B-ELISA for BTV Antibodies

The enzyme substrate was a key reagent in the A/B-ELISA. Optimal sensitivity of the assay depends upon several variables associated with the substrate solution. The concentration of hydrogen peroxide (H_2O_2) and chromogen used in the substrate solution were critical as were the pH of the substrate solution and the incubation time allotted for the enzyme-substrate reaction. Previous studies indicated that the optimal concentrations of H_2O_2 and OPD, the chromogen used in the present study, were 2.0 mM (0.03%) and 2.2 mM (0.8 mg/ml) respectively (138). Less favorable results were obtained in trial procedures of this study when concentrations other than those were used. The H_2O_2 solution should be prepared from fresh concentrated stock just prior to use. The laboratory practice was to procure a new bottle of H_2O_2 every 4 weeks. The substrate solution should have a pH of 4.5 and the reaction incubation time appeared to be optimal at 30 minutes. The

relatively short incubation time did not necessitate the incubation of the plates in the dark nor the stopping of the reaction with H_2SO_4. On 2 trials, the acid stopped plates were held for 24 to 36 hours and read again. At these times there was less than 2.0% variation in the binding ratios from the initial reading.

In addition, the plate rinsing procedure was found to be critical to the A/B-ELISA. Rinsing the plate after each incubation step was necessary to remove any loosely-bound or totally unbound protein from the wells. Tween 80 was added to the rinsing solution to aid in the removal of nonspecifically bound protein. However, this measure might prove to be detrimental to the assay if the number of rinsings was excessive resulting in partial or complete removal of the specifically bound protein from the wells. Seven rinses were determined to be optimal when using the sodium chloride-Tween 80 solution and the semi-automatic plate washer for all rinsing steps except following the application of the ABC reagent. The number of rinses was inceased to 14 following that step to insure that all the unbound enzyme conjugate was removed from the wells.

The semi-automatic microtiter plate washer was a tremendous improvement towards convenience and time savings with the A/B-ELISA. However, caution has to be exercised when the plate washer was used to insure that all wells were rinsed with equal force and with equal volumes of

rinse solution. Uneven rinsings could significantly alter the results of the assay. To minimize this problem in the present study, sera were usually run in triplicate and the mean of the 3 absorbance values taken as the absorbance of that particular serum sample.

Discussion

The purpose of this study was to determine whether the A/B-ELISA could be applied for the detection of BTV antibodies in a mouse model system and the optimal conditions necessary for such an application. It was demonstrated by the present study that A/B-ELISA, utilizing BTV 11, BTV 13 and BTV 17 antigens, is an effective assay for the detection of antibody to BTV.

Two different methods of antigen preparation were evaluated with the assay. The first method, in which BTV infected cell cultures were utilized as the source of viral antigen, although acceptable for the preparation of agar gel immunodiffusion (AGID) antigen, did not render interpretable results with A/B-ELISA (Tables 2, p 40 and 3, p 50). These results can be explained by a lack of BTV antigen from this type of virus-cell mixture being bound to the microtiter plate. Several explanations for this observation should be considered. Inadequate binding in that all, or some lesser amount, of the bound antigen was rinsed away during the remaining steps of the assay

could account for such haphazard results. Alteration of the antigen during processing might also have rendered it incapable of the high affinity binding necessary for it to withstand the multiple rinsings of the assay without being dislodged. If the antigen preparation was such that the final antigen solution was hydrophobic, very little antigen binding would be expected. A desired method of analyzing the problem would have been to have compared the BTV protein/total protein amounts and ratios for the tissue culture and mouse brain source of antigen. However, since the mouse brain virus would not grow readily in cell culture and the tissue culture virus was not lethal for mice (before adaptation by blind passage), the titer of infectious virus could not be compared in the same system. In conclusion, antigen binding was considered to be the basis for the uninterpretable results obtained with the cell culture antigen. However, due to the successful results obtained with the second method of BTV antigen preparation, i.e., the mouse brain antigen, the problem was not investigated further.

Concerning antigen coating buffers, carbonate (pH 9.0 and 9.6), PBS (pH 7.6, 8.0 and 8.5), acetate (pH 8.2) and glycine (pH 10.0) were used with the A/B-ELISA. Most of these buffers have been used effectively with different antigen systems and ELISA (17, 128, 139, 160, 166). In the present study, the buffers were tested with the BTV system

to determine which would give the best results with the A/B-ELISA. Better binding ratios, i.e., separation between positive and negative sera, were obtained with the carbonate buffers, especially at a pH of 9.6 (Fig. 4-6, p 44-46). These findings were in agreement with those of a previous study in which carbonate buffer was found to preserve the integrity and infectivity of BTV for up to 48 hours (78).

Theoretically it is possible for proteins other than specific antibody, i.e., immunoglobulin aggregates, immune complexes, and nonspecific antibody, to adhere to sites on the microtiter plate not occupied by the antigen. Such nonspecific binding might also be related to the absolute amount of immunoglobulin, both specific and nonspecific, present in the serum sample (139). When the serum sample is incubated in the microtiter well, all proteins present in the sample have an opportunity to adhere to available sites and over a period of time all the binding sites in the well will be bound by one protein molecule or another. For this reason, the higher the concentration of nonspecific immunoglobulin in a serum sample the greater the opportunity for nonspecific or background binding to take place. Nonspecific binding, no matter what the cause, could reduce the sensitivity and specificity of the assay and create the possibility for false positive results. Even though the A/B-ELISA was designed to minimize

nonspecific binding, results of the initial experimental assays in this study indicated that considerable nonspecific staining did occur with all antigen-antibody dilutions examined (normal sera values, Table 2, p 40). It has been reported (139, 160) that nonspecific binding can be reduced by adding nonspecific protein, such as BSA, and nonionic detergent, such as Tween 20 or Tween 80, to the diluents for sera and conjugate. The nonspecific protein is considered to occupy all available binding sites not taken up by specifically bound protein. The detergent aids in the removal of bound protein which has less affinity for the solid phase than does antibody which is specifically bound to antigen. In the present study several different combinations of buffers (PBS and Tris), nonspecific proteins (BSA, gelatin, NHS, FCS and TPB), and detergent (Tween 20) were tested for use with A/B-ELISA. The best results were obtained with the PBGT solution (Fig. 7, p 48). This solution was selected as the diluent for all reagents other than antigen in the A/B-ELISA.

The data (Fig. 9-11, p 52-54) clearly showed the group specificity of the A/B-ELISA for BTV. There was cross-reaction between the 3 BTV serotypes examined which was an indication of the assay's usefulness in detecting antigens common to all BTV serotypes. The BTV specificity of the assay was also demonstrated by the similarity of the binding ratios calculated (binding ratios $\cong$ 1.00) when

normal mouse serum and hyperimmune mouse anti-SRBC serum were assayed against each of the 3 BTV antigens. It was also notable that absorbance values obtained from wells containing either BTV 13 antigen or anti-BTV 13 serum are consistently lower than those obtained from wells containing either antigen or antiserum from the other 2 BTV serotypes (Fig. 9-11, p 52-54). These findings might directly result from the amount of viral antigens present in the respective viral suspensions as they were originally obtained. After mouse adaptation procedures, the MLD_{50} of stock P_5/BTV 13 mouse brain suspension was approximately 2 logs less than that of stock P_5 mouse brain suspensions of BTV 11 and BTV 17. This in itself could result in lower levels (titers) of BTV 13 antigen and antibody utilized in the assay as the stock P_5 mouse brain suspension was used in the preparation of both antigen and antibody.

In the present study A/B-ELISA was evaluated as a method for the detection of antibodies to BTV. The present results allowed for the differentiation of bluetongue-positive and negative sera. The assay procedure was rapid and easy to perform with the pre-titrated staining kit (Vectastain ABC Kit), and lends itself well to the detection of antibodies against BTV in the mouse model system.

CHAPTER IV

APPLICATION OF AVIDIN/BIOTIN-PEROXIDASE COMPLEX (ABC) IMMUNOPEROXIDASE STAINING TECHNIQUE FOR DETECTION OF BLUETONGUE VIRUS ANTIGEN IN MOUSE TISSUES

Introduction

The basis of histopathological diagnosis lies in the interpretation of microscopic morphology of lesions. Conventional histochemical stains have been and continue to be essential aids in the interpretation of cytological and anatomical changes, but they lack the specificity of newly developed immunological reagents for recognizing individual cells infected with a viral agent. Immunohistochemical techniques are considered to be a relatively young branch of histochemistry, although antigens were first located in tissue sections with fluorescein-labeled antibodies over 40 years ago (32). During succeeding years, immunofluorescent methods became widely used in biomedical research as well as in diagnostic pathology. This increased interest in demonstrating specific antigens precipitated an increased demand for a satisfactory alternative labeling system, primarily to avoid the need for specialized equipment, i.e., a microscope with ultraviolet light capabilities, and the problems of stain deterioration and poor morphologic detail of the immunofluorescent preparations (155).

A promising alternative to immunofluorescent methods, the immunoenzymatic technique was first reported in 1966 (113). After the initial introduction, immunoenzymatic techniques were quickly demonstrated to be as sensitive if not more so than the corresponding immunofluorescent procedures (149). Tissue sections stained with immunoenzymatic techniques kept well and could be counterstained with conventional histochemical stains. In addition, the sections retained morphologic qualities of paraffin-embedded tissues and could be examined with routine light microscopy. This technique utilized enzyme-antibody conjugates to detect, localize and quantitate antigens in tissues. A number of procedures employing various cross-linking agents were used to prepare the necessary conjugates. Generally, these conjugates were prepared by covalently coupling the enzyme marker to the antibody (5, 6, 9, 114, 156), but some noncovalent coupling procedures were also used (8, 105, 149).

Recently, the noncovalent, but exceptionally strong binding characteristics of avidin and biotin (dissociation constant: 10^{-15}M), have been used to link enzyme markers to antibody in immunoenzymatic assay procedures (29, 55, 74, 76, 77).

Avidin is a 68,000 molecular weight glycoprotein found in egg white. Biotin is a small molecular weight vitamin widely distributed in mammalian tissues (74, 173). The

avidin/biotin affinity is significantly higher than that of most antibodies for antigen, and unlike antigen-antibody interactions, the avidin/biotin interaction is essentially irreversible. The avidin/biotin system possesses the advantage over conventional immunoenzymatic methods of not requiring any high molecular weight protein-protein conjugates. The system involves only the preparation of biotin-labeled protein. Additional features which enable the system to be effectively exploited are found in the ability of avidin to bind 4 molecules of biotin and the ability of most proteins, including enzymes to conjugate with several biotin molecules.

Of the recently developed avidin/biotin enzyme immunoassays, an indirect bridged technique utilizing a preformed avidin/biotin-peroxidase complex (ABC) has been reported to be less time-consuming and more sensitive than other methods (74). The ABC (Fig. 1, p 27) is created by incubating excess avidin with biotin-labeled peroxidase. During this procedure, avidin acts as a bridge between molecules of biotin-labeled peroxidase. However, at the same time, biotin-labeled peroxidase molecules which contain several biotin moieties also function as a link between avidin molecules, resulting ultimately in a "lattice" possessing several peroxidase molecules. When bound to tissue by means of the biotin-labeled secondary antibody, the complex stains intensely (74). Tissue

preparation techniques were very critical to early immunocytochemical methodology. Antigens were best preserved for visualization with immunofluorescent staining procedures in frozen (cryostat) sections. Possibly because most, if not all, antigenic determinants are destroyed during the harsh fixation and embedding procedures utilized in routine tissue processing, the early immunoperoxidase staining techniques were of inadequate sensitivity to gain wide acceptance. However, the development of highly sensitive immunoenzymatic assay systems such as the ABC method has often made the preparation of tissues for immunohistochemical staining less critical. Even so, some antigens may still require special tissue processing before they can be visualized. Bluetongue virus (BTV) antigen, which was readily visualized in frozen sections with immunofluorescent methods, was not visualized, by similar or modified techniques, in tissue sections processed for standard paraffin embedding (CA Holmberg, personal communication). Because BTV is heat labile, routine paraffin embedding procedures in which the temperature often exceeds 60°C, might destroy most, if not all, of the antigenic determinants of the BTV tissue antigen present. Therefore, the detection and localization of BTV antigen in paraffin embedded tissues might only be possible by utilizing a highly sensitive assay in conjunction with tissue processing utilizing temperatures

lower than those of routine paraffin embedding procedures. The ABC immunoperoxidase staining technique as described above is reported to be extremely sensitive (29, 55, 74, 76, 77), however it has not been applied to the detection of BTV antigen in tissue sections. Also, an acetone-paraffin embedding procedure, in which low melting-point paraffin (50-53°C) is used, has recently been described for enzyme immunohistochemical assays (30). It was the purpose of this study to determine if the ABC immunoperoxidase staining technique could be applied in conjunction with the acetone-paraffin embedding procedure for detection and localization of BTV antigen in mouse tissues. A newly described long-term incubation procedure (145) was assessed, and its advantages over the more conventional short-term incubation methods were discussed. Several methods of enhancement or intensification of the enzyme-substrate reaction product were also tested.

Materials and Methods

Mouse Adaptation of Virus

The 4 American serotypes of BTV [low passage isolates in Oxylated-Carbol-Glycerin solution (OCG)-treated sheep blood] were obtained from Dr. Stewart McConnell (Department of Veterinary Microbiology and Parasitology, Texas A & M University, College Station, TX) and adapted to suckling

mice as previously described in Chapter I.

Preparation of Antisera

Thirty adult C57 mice were divided into 3 groups of 10 mice each. Each mouse in one group was inoculated intraperitoneally (IP) with 0.5 ml (4.45×10^4 MLD_{50}) of thawed and undiluted P_5/BTV 11 mouse brain suspension once a week for a total of 3 inoculations. Mice in the second and third groups were inoculated in a similar manner with P_5/BTV 13 (1.41×10^3 MLD_{50}) and P_5/BTV 17 (1.41×10^5 MLD_{50}) mouse brain suspensions, respectively. Two weeks following the third inoculations, all mice were exsanguinated and their serum pooled as per individual groups, aliquoted, and stored at -70°C as stock anti-BTV sera. Normal control serum obtained from nonimmunized C57 mice was aliquoted and stored at -70°C as stock normal serum.

Tissues and Fixation

Six suckling (1- to 3-day old) C57 mice were inoculated intracerebrally with 0.02 ml (1.78×10^3 MLD_{50}) each of a thawed, undiluted stock P_5/BTV 11 mouse brain suspension. Six additional suckling C57 mice were inoculated intracerebrally with 0.02 ml DMEM and served as controls. Following inoculation the 2 groups of mice were housed separately with their respective dams. When the

inoculated mice became moribund, generally 3 to 6 days post-inoculation, both groups of mice were decapitated, and an incision was made in the skin and cranium along the dorsal midline of the head. The skin was reflected, and the cranial vault carefully opened, and the entire head emersed in 10% buffered formalin for 48 hours. Following fixation, 2 cross-sections of approximaely 3mm in thickness, were cut, 1 from the mid-cerebellar region and the other from the hippocampus and mid-cerebral region, and processed for histochemical [hematoxylin and eosin (H&E)] and immunohistochemical staining.

Tissue Processing

The processing of tissue for immunoperoxidase study was performed with modification of previously described acetone-paraffin methods (30). After fixation, tissues were rinsed in running tap water for 8 to 10 hours before being loaded into an automatic tissue changer (Autotechnicon, Technicon Ltd., Swords Co., Dublin, Ireland) programmed as follows: 50% acetone (30 min. X 1); acetone (30 min. X 3); xylene (45 min. X 3) - all solutions held at room temperature and finally, 52°C melted paraffin baths (90 min. X 2). The tissue blocks were equilibrated at room temperature prior to cutting 5 to 6 um sections. Egg albumin was used to ensure proper adhesion of the sections to glass slides. The slides were dried overnight

in a 55°C oven and were stored in dustproof slide boxes until needed.

Avidin and Biotin Reagents

Stock solutions of affinity-purified biotinylated horse anti-mouse IgG, normal horse serum, avidin, and biotinylated horseradish peroxidase were obtained as a kit (Vectasin ABC Kit, Vector Laboratories, Inc., Burlingame, CA). All reagents supplied with the kit were diluted to working strength as per the kit instructions (described in Chapter I).

Enzyme Substrate Solution

Diaminobenzidine [3,3' - diaminobenzidine tetrahydrochloride (DAB), Litton Bionetics, Inc., Kensington, MD] was the substrate chromagen recommended for use with the ABC staining procedure (29, 55, 74, 76, 77). DAB was supplied as a lyophilized material, 5 mg per vial. Reconstitution to a stock 0.5% solution was accomplished by adding 1.0 ml of 0.05M Tris-HCl buffer (Sigma Chemical Co., St. Louis, Mo), pH 7.4. The substrate solution was prepared immediately before use, and consisted of 0.05% DAB and 0.01% H_2O_2 in 0.05M Tris buffer, pH 7.2.

Staining Intensification Methods

Three recently reported means of enhancing or intensifying the reaction product of peroxidase and DAB were tested with the ABC staining procedure (61, 152, 153, 173). Two of the methods involved post-treatment of the tissue sections for 2 minutes with osmium tetroxide (OsO_4) (153), or for 5 minutes with 0.5% cupric sulfate ($CuSO_4$) (61, 173), respectively. The third method involved the addition of 0.01M imidazole, pH 7.4, to the substrate solution (152). The imidazole/DAB substrate consisted of 0.04% DAB, 0.015% H_2O_2, and 0.01M imidazole in 0.05M Tris-HCl buffer pH 7.4.

Suppression of Endogenous Peroxidase Activity

A number of methods have been described for inhibiting endogenous peroxidase to avoid false positive results in immunoperoxidase assays due to its presence in tissues. A modification of the periodic acid-borohydride method (65) and the use of extended incubations in various concentrations of H_2O_2 in methanol were investigated for that purpose in the study.

The periodic acid-borohydride method involved incubating the sections in 7.5% H_2O_2 in distilled water for 5 minutes. In the present study, absolute methanol was substituted for distilled water in this initial step of the procedure. Following a tap water rinse, the tissues were

incubated for an additional 5 minutes in 2.8% periodic acid and again rinsed in tap water. The sections were then incubated in 0.02% potassium borohydride for 2 minutes. A tap water rinse was followed by a series of 4 - 5 minute washes in buffer.

When H_2O_2 in methanol was used alone to inhibit the endogenous peroxidase activity, the sections were incubated in the solution for 20 minutes followed by a 20 minute wash in buffer.

Routine ABC Staining Procedure

The procedure used was essentially that as supplied with the ABC staining kit.

Before staining, the tissue sections were deparaffinized, hydrated through xylene and graded alcohol, and rinsed for 5 minutes in distilled water. The sections were then incubated in 0.3% H_2O_2 in methanol for 30 minutes at room temperature (RT) to inactivate endogenous peroxidase activity in the tissue, and washed in buffer for 20 minutes. Following the buffer wash, the sections were incubated for 20 minutes with 3% normal horse serum (NHS), to block any species-dependent (i.e., horse) antigenic determinants in the tissue. Excess NHS was blotted from the slides before several drops of primary antiserum (anti-BTV 11), diluted 1:200 in buffer, were added to each section. Following a 30 minute incubation at RT, sections

were washed for 10 minutes in buffer and incubated for an additional 30 minutes with secondary antibody, (biotinylated horse anti-mouse IgG), diluted 1:250 in buffer, at RT. A 10 minute wash of the sections in buffer preceeded a 30 to 60 minute incubation with the ABC reagent (prepared by adding 20 ul avidin and 20 ul biotinylated horseradish peroxidase to 10 ml buffer).

Following incubation with ABC reagent, sections were washed for 10 minutes in buffer and incubated for 4 to 5 minutes in DAB solution which consisted of 0.01% H_2O_2 and 0.05% DAB in 0.05M Tris buffer, pH 7.2. Sections were then washed for 5 minutes in tap water, counterstained, cleaned and coverslipped.

Solutions for Long-Term Staining Procedure

Tris/phosphate buffer (TPB). The same buffer (0.04M Tris and 0.01M phosphate) which consisted of 5.0 g Tris (hydroxymethyl)-aminomethane, 2.26 g Na_2PO_4 $7H_20$, 0.25g $NaH_2PO_4.2H_20$, and 7.0 g NaCl in 1 liter of deinoized water, adjusted to pH 7.8, was used to prepare the carrageenan/triton diluent solution (described below) and as a wash fluid for rinsing tissue sections between the various steps of the staining procedure.

Carrageenan/Triton Solution (CTS). The solution consisted of 0.5% (by weight) Triton X 100 (Sigma Chemical Co., St. Louis, MO), and 0.7% (by weight) lambda carrageenan (Sigma) dissolved in TPB. As a detergent, Triton X 100 was utilized to aid the penetrability of large molecular weight molecules (such as antibody) into the tissue sections. Lambda carrageenan, a non-jelling seaweed gelatin, was added to the solution to reduce nonspecific staining in the immunoenzymatic procedures.

Long-Term Incubation and Staining Procedure (LTI)

The procedure was a modification of several previously described incubation and staining procedures (55, 74, 76, 145, 150). It employed the immersion of tissue sections on whole glass slides into jars of immunoperoxidase staining reagents which permitted the use of long incubation times without the sections drying out. The long incubations often enhanced the sensitivity and aided in the reproducibility of the staining obtained (145).

Prior to staining with the LTI, sections of BTV-infected and non-infected brain from suckling C57 mice were depariffinized, rehydrated, and washed twice for 10 minutes each in fresh changes of TPB. The sections were then treated with 0.03% H_2O_2 in methanol for 20 minutes, followed by a 20 minute wash in TPB. After washing, the sections (on whole glass slides) were placed in a Coplin

staining jar filled with 3% NHS in CTS and incubated for 20 minutes at RT. Excess NHS was blotted from the slides before they were placed in a Coplin staining jar filled with primary antisera (1:200 dilution of mouse anti-BTV 11 in CTS) and incubated for 48 hours at 4°C.

Following incubation in primary antiserum, the sections were washed in 3 changes of fresh TPB over a total time of 10 minutes, and immersed for 60 minutes at RT in a Coplin staining jar filled with secondary antibody (1:250 dilution of biotinylated horse anti-mouse IgG in CTS). Subsequently, they were again washed in 3 changes of fresh TPB and immersed in a jar of diluted ABC reagent (prepared as per the ABC staining kit instructions with CTS as the diluent), and incubated for 60 minutes at RT.

Following incubation with ABC reagent, sections were washed in 4 changes of fresh TPB over a total time of 20 minutes, and were placed in a moist chamber and covered with several drops of freshly prepared DAB solution (either standard DAB solution or the imidazole/DAB solution) and incubated for 3 to 4 minutes at RT.

Following the DAB incubation, sections were quickly rinsed with tap water and counterstained in 1.0% methyl green for 30 minutes. If the OsO_4, or the $CuSo_4$ intensification methods were to be used, they were inserted between the DAB incubation and the counterstaining procedure. After counterstaining, sections were rinsed in

tap water and dehydrated through graded alcohol, and xylene, and coverslipped in permount.

Controls

BTV-infected and non-infected control sections of brain were placed adjacent to each other on a glass slide in order that both received the same treatment during the staining procedure. Also, controls for the specificity of the ABC staining method were performed. These were as follows:

1. Replacement of the specific primary antiserum (anti-BTV 11) with normal mouse serum.
2. Replacement of the specific primary antiserum with a second primary antiserum prepard in the mouse, but raised against an antigen unrelated to the 1 under investigation. Mouse anti-sheep red blood cell (SRBC) serum was used as the second primary antiserum.
3. Deletion of the secondary antibody incubation (biotinylated horse anti-mouse IgG).
4. Deletion of the ABC reagent incubation.
5. Deletion of the primary and secondary antibody incubation.
6. DAB solution alone.

Results

The clinical signs, and the character of the histologic lesions observed in the brains of suckling mice infected with BTV in this study, were very similar to those previously reported (107). Vascular lesions involving the small blood vessels of the brain were the predominant morphologic changes observed, and varied from subtle endothelial cell swelling and margination of neutrophils to

severe vasculitis, perivasculitis and vascular thrombosis. Other lesions were usually closely associated with the vascular changes, and were indicative of those pathlogic responses usually associated with primary damage to the cerebral vasculature such as ischemic neuronal degeneration (174), coagulation necrosis, edema, and hemorrhage.

Immunoperoxidase Staining

Routine ABC procedure. Sections of BTV-infected, and non-infected, suckling mouse brain were stained with the ABC staining procedure as described in the materials and methods sections above. The procedure failed to satisfactorily demonstrate BTV antigen when either 30 minute or 60 minute incubations were the ABC reagent used. Very little detectable, specific staining was observed in the sections, even though lesions compatible with those of encephalitis were clearly evident (Fig. 12 and 13). The experiment was repeated several times, each time modifying the procedure to include 1 or more of the intensification methods decribed above, but with limited success. Better specific staining was demonstrated when the procedure was modified to include the imidazole and the $CuSO_4$ intensification methods with a 60 minute ABC reagent incubation. However, the degree of staining produced by the procedure remained less than desirable for the purpose of the study.

Fig. 12. Perivascular hemorrhage, edema and neuronal necrosis in cerebral cortex of bluetongue virus (BTV) infected suckling mouse. Routine avidin/biotin-peroxidase complex (ABC) staining method, methyl green counterstain, 130X.

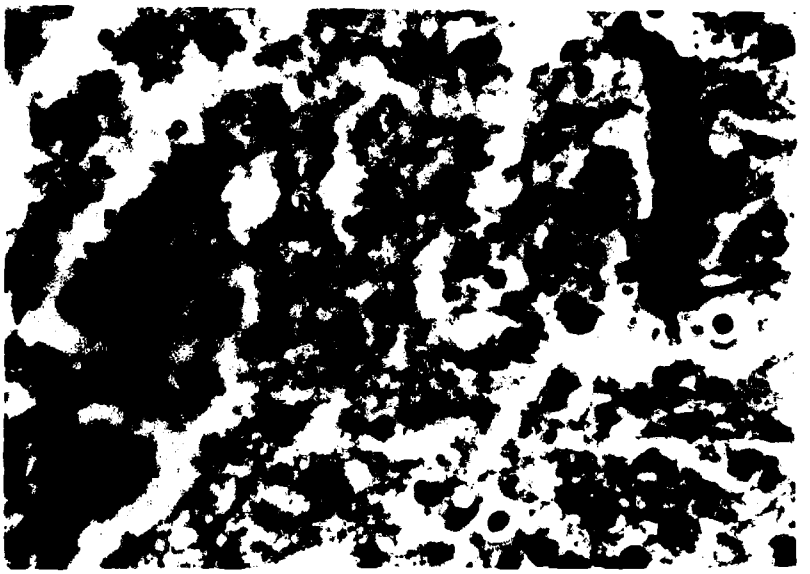

Fig. 13. Endothelial cell hypertrophy, vascular sludging and perivascular edema in cerebral cortex of bluetongue virus (BTV) infected suckling mouse. Necrotic neurons (N) are present in the neuropil. Endothelial cells (E). Routine avidin/biotin-peroxidase complex (ABC) staining method, methyl green counterstain, 375X.

Long-term incubation period (LTI). The extended incubation of the brain sections in primary antiserum diluted in CTS greatly enhanced the sensitivity of the staining achieved with the ABC procedure. BTV antigen was readily demonstrated within the cytoplasm of neurons, astrocytes, and endothelial cells throughout the affected areas of the brain (Figs. 14-18). Antigen was also observed in the cytoplasm of endothelial cells of vessels considerable distances away from the areas of inflammation, including vessels in the cerebellum.

In the cytoplasm of cells, the material representing the presense of BTV antigen, i.e., the DAB reaction product was granular, variable in size (often punctate), distributed randomly, and stained dark brown. Similarily staining granular material was also observed in the lumens of many of the inflammed vessels, and was considered to be blood platelets. Brown-staining material was not observed in the blood vessels of non-infected control animals.

Although the long-term incubation method satisfactorily demonstrated BTV antigen in the brain sections, modifications were attempted to determine the optimal conditions and reagents necessary to obtain maximal sensitivity with the assay for BTV antigen. Through a series of experiments, it was determined that the incubation of sections in primary antiserum could be shortened to 48 hours without losing appreciable staining

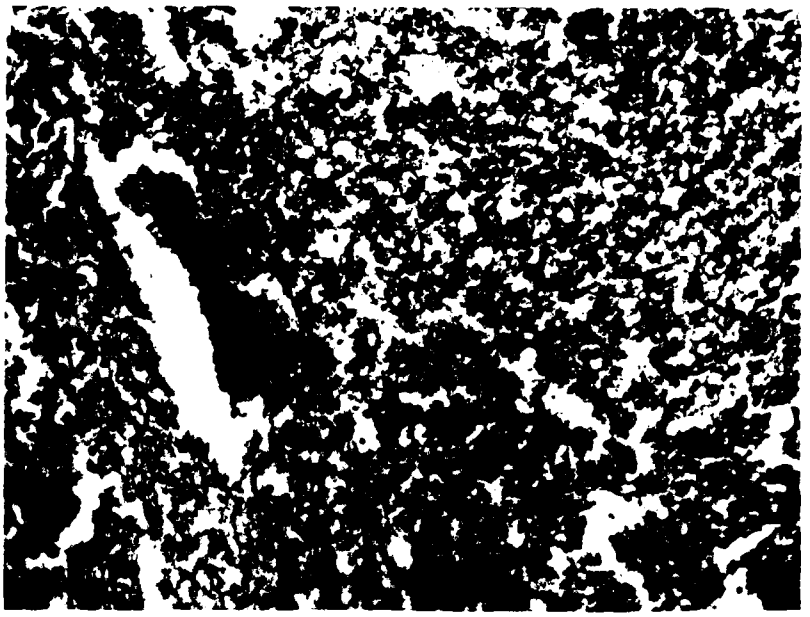

Fig. 14. Section of cerebral cortex of bluetongue virus (BTV) infected suckling mouse stained by the long-term incubation-avidin/biotin-peroxidase complex (ABC) method of staining. Numerous darkly stained immunoperoxidase positive cells are scattered throughout the field, 130X.

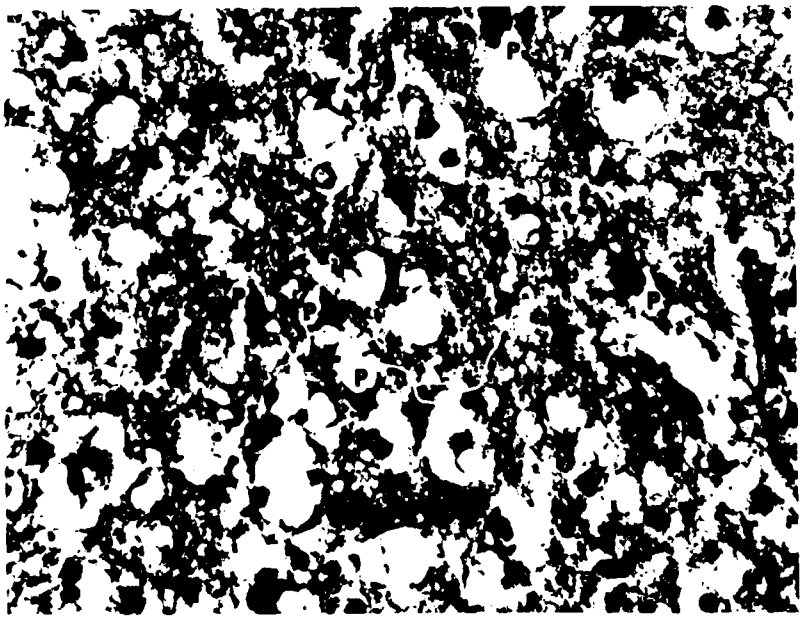

Fig. 15. Higher magnification of Fig. 14, demonstrating status spongiosus and positive immunoperoxidase stained neurons (P). Long-term incubation-avidin/biotin-peroxidase complex (ABC) method of staining, 340X.

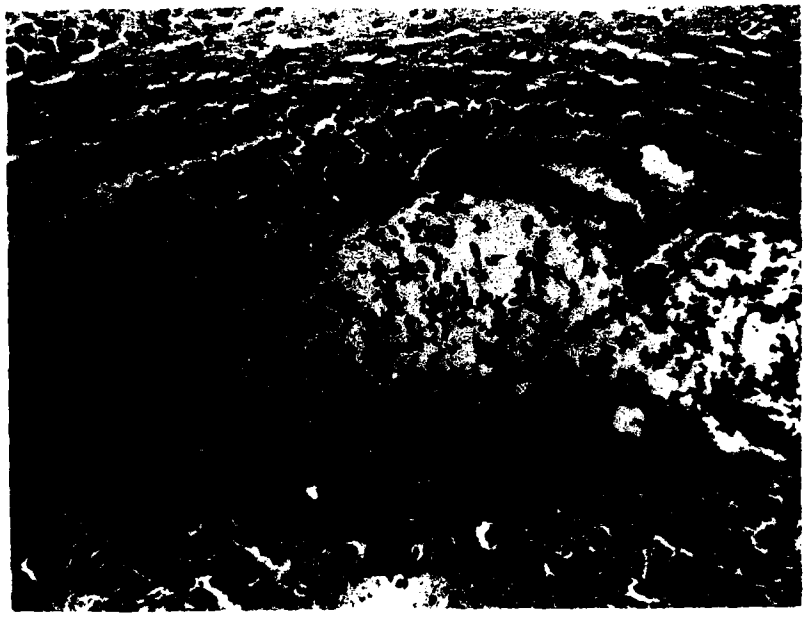

Fig. 16. Necrosis and edema of hippocampus in brain of bluetongue virus (BTV) infected suckling mouse. Long-term incubation-avidin/biotin-peroxidase complex (ABC) method of staining, 130X.

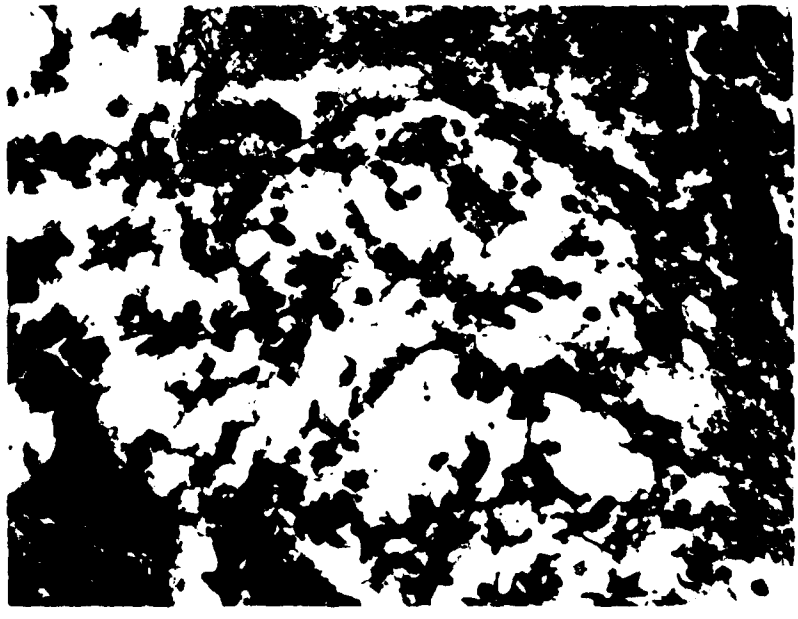

Fig. 17. Higher magnification of Fig. 16, long-term incubation-avidin/biotin-peroxidase complex (ABC) method of staining, 340X.

Fig. 18. Section of cerebral cortex of bluetongue virus (BTV) infected suckling mouse stained by the long-term incubation-avidin/biotin-peroxidase complex (ABC) method of staining. Positive staining of capillaries and small blood vessels (arrows), 133X.

intensity, so long as the imidazole and $CuSO_4$ intensification methods were utilized in the procedure.

The battery of controls indicated that very little nonspecific binding occurred that could not be differentiated from the specific reaction. However, the DAB solution along, or in conjunction with ABC reagent, resulted in considerable background staining. The intensity of this background staining varied with incubation time for the DAB substrate, i.e., the longer the incubation, the darker the background staining. Nonetheless, this background staining was strikingly uniform throughout the tissue section and almost always less intense than the specific DAB reaction product. In a well prepared section, the 2 types of staining were easily distinguished from each other.

The long-term incubation-avidin/biotin-peroxidase complex (ABC) staining technique is summarized schematically in Fig. 19. The presence of BTV antigen in the brains of suckling mice previously inoculated with BTV 11 was adequately demonstrated with this technique. Once the procedure was finalized, the technique was convenient and allowed for consistent day-to-day staining of multiple tissue sections.

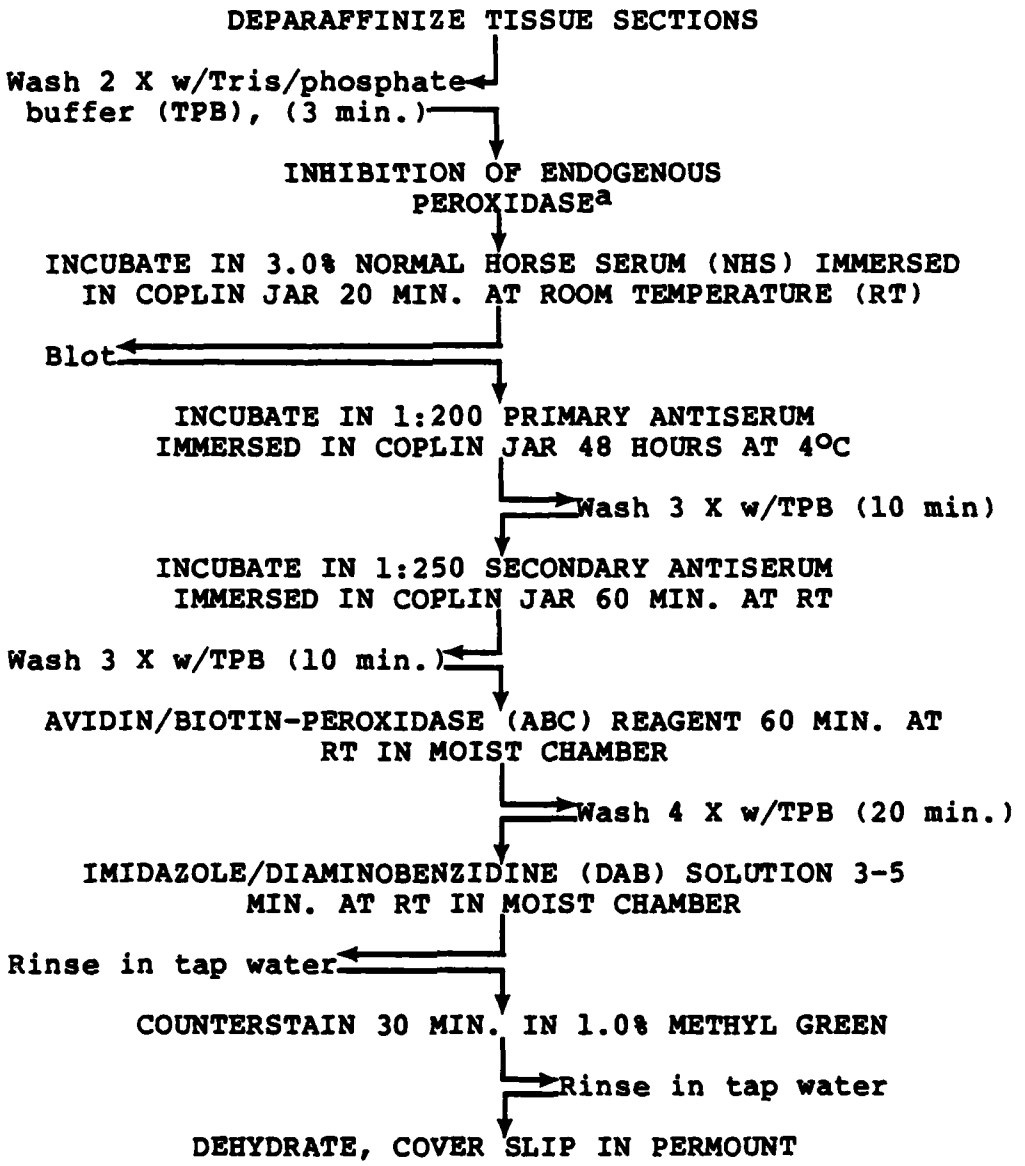

Fig. 19. Schematic representation of the long-term incubation-avidin/biotin-peroxidase complex method of staining.

[a]Periodic acid-borohydride method

Discussion

The present study was designed as an attempt to demonstrate BTV antigen in mouse tissues, utilizing immunoperoxidase techniques. For this purpose, neonatal mice inoculated intracerebrally were used as the virus infected tissue substitute.

Despite the reported success of the ABC immunoperoxidase staining technique, recommendations suggested by the manufacturer of the ABC Kit were not suitable for identifying BTV antigen in mouse brain. Only after modification of the procedure to include intensification methods with imidazole and/or $CuSO_4$ were significant amounts of specific immunostaining of BTV antigen visualized in the tissues. This discrepancy might be largely attributed to tissue fixation which has been strongly implicated as the more important factor responsible for the lack of immunostaining (168). Other factors might include solubilization (75), alteration, or destruction of the antigen during tissue processing, or during the methanol-H_2O_2 treatment (65). The fact that BTV antigen was clearly demonstrable in sections cut from the same tissue blocks when the ABC technique was used in conjunction with the LTI indicated that recognizable BTV antigenic determinants remained in the tissue after fixation, processing and staining. Another possible explanation for the lack of success with the routine ABC

technique might be in the physical nature of the ABC reagent itself. By definition, the complex is an extraordinarily large structure which would encounter great difficulty in cellular penetration. The routine ABC technique employs no modification such as trypsinization (35), freezing and thawing (115), or detergent treatment (57) to facilitate the tissue penetration of its immunoreagents. Conversely, the LTI method utilizes a detergent solution, CTS, as a diluent for all the immunoreagents. In addition, the exceptionally long incubation times made possible by immersing the whole slide into antiserum diluted in CTS probably enhanced staining equilibrium. Because an antiserum actually contains a hetercgeneous population of antibodies that have differing affinities for the antigen, a certain minimum time would be required for the most avid antibodies, which must compete with the less avid antibodies in the antiserum, to bind the available antigen and come to equilibrium. This has been shown to be the case with radioimmunoassay of small peptides, where it often requires a number of days for the antisera to reach equilibrium when incubated with radioactively labeled peptide (63, 144). By increasing the incubation time in primary antiserum, a greater number of antibodies with a higher affinity become bound to the tissue antigen. These more avid antibodies are less likely to be washed away during the subsequent steps of the

procedure, thereby, increasing the sensitivity of the assay.

Other advantages of the LTI procedure are also directly related to the method of incubation. The immersion of the entire tissue attached to a glass slide into a jar of diluted antisera kept the sections from drying out during incubation. Also, there would be no appreciable changes in the concentration of the antisera due to evaporation, and the problem of choosing a level site for the incubation, so that the antisera would not run off of the sections, is not encountered.

A disadvantage of the procedure, as compared to the conventional incubation procedure in which slides are placed flat in a moist chamber and only the tissue sections covered with drops of antisera, is the initial cost of preparing the jars of antisera. However, once prepared, if stored at 4°C, and if incubations are conducted at 4°C, the antisera has been reported to be stable for years (145); thus the initial reagent costs are regained several times over. Because a small amount of antiserum is removed when removing slides from the jars (the dripping of antiserum from slides back into the jar should be allowed), 10 to 20 ml of antiserum, diluted identically as that already in the jar, should be added as needed.

Also, due to the high cost of commercial ABC reagents, and because ABC might dissociate if stored as a complex for any length of time, working dilutions of the enzyme complex

were not made up and stored in Coplin jars, as were the other immunoreagents. Rather, the ABC was carried out in the conventional moist chamber fashion.

In addition to the LIT procedure, the sensitivity of the assay appeared to also be increased by the incorporation of the imidazole modification. Previous reports had indicated that the reaction of peroxidase with DAB and H_2O_2, was considerably more intense at pH 5.0 than at pH 7.6 (103, 154, 170). However, later reports demonstrated perinuclear staining artifacts when the low pH was used. The artifacts did not occur when the reaction was performed near a neutral pH, but the staining was less intense (135). Subsequently, it was shown that at pH 7.0 to 7.8 the rate of oxidation of DAB and therefore, the intensity of the reaction, could be enhanced several-fold by the addition of 0.01M imidazole (152). By utilizing the imidazole modification with the assay, optimal intensity of the peroxidase-DAB/H_2O_2 reaction should be expected; and this was indeed an observation of this study.

Cupric sulfate ($CuSO_4$) had previously been used with immunoperoxidase techniques as a means of darkening the brown DAB reaction product (156, 173). Although the method did aid in the intensification of the reaction with the routine ABC staining technique, its usefulness was questionable when combined with the LTI and the imidazole modifications, which themselves enhanced staining intensity.

If utilized with the LTI, and the imidazole method, the $CuSO_4$ modification added an additional step to the assay procedure, but added very little towards the visualization of the DAB reaction product.

A number of methods have been used to inhibit endogenous peroxidase in tissue sections. Excellent results, including the inhibition of endogenous peroxidase in eosinophils (polymorphonuclear leukocytes in which peroxidase enzymatic activity is difficult to block), have been reported with the periodic acid-borohydride method (65). During modification of the ABC technique for use with mouse tissue, the periodic acid-borohydride method was compared with a procedure utilizing various concentrations of H_2O_2 in methanol. Results demonstrated that the periodic acid-borohydride treatment was the better of the 2 methods. It significantly decreased the amount of background staining in the tissue sections and also provided a crisp morphologic definition typical of periodic acid-treated sections.

The results of the present study also indicated that formalin fixation in conjunction with acetone dehydration (as opposed to formalin fixation and routine ethanol dehydration) and low melting-point paraffin embedding, was a satisfactory method of processing mouse brain for the localization of BTV antigen with the modified immunoperoxidase techniques described above. In addition,

this method of processing also proved satisfactory for conventional histochemical staining. The anatomical detail of H&E stained tissue sections processed in this manner was almost as good as that of sections processed by routine methods. However, an initial problem was encountered with the processing procedure when attempts were made to cut sections from the tissue blocks. Chilling of the blocks prior to sectioning (a common practice with routinely processed brain tissue) resulted in cracked or shattered sections that were often unusable. This undesirable feature was virtually eliminated by allowing the tissue blocks to equilibrate with room temperature prior to sectioning.

CHAPTER V

IMMUNOLOGIC AND PATHOLOGIC CONSEQUENCES OF SECOND HETEROTYPIC BLUETONGUE VIRUS INFECTION IN MICE

Introduction

Bluetongue virus (BTV) is an arthropod-borne agent known to infect many ruminant species with variable disease expression (20, 69, 70, 158). The virus is classified in the genus Orbivirus, family Reoviridae (15, 25, 127, 165), and is antigenically related to the epizootic hemorrhagic disease virus (EDHV), another orbivirus known to infect ruminants in the United States and Canada (15, 165). Currently, there are 20 serotypes of BTV recognized worldwide. Four, BTV 10, BTV 11, BTV 13 and BTV 17, are present in the United States with 1, BTV 17, considered unique to North America (10).

Bluetongue virus infection is widespread in nature, although disease expression is observed only as limited outbreaks in certain herds or flocks (11, 20, 124). In addition, disease manifestation is extremely variable, and clinically inapparent carrier states frequently occur in ruminants. Several factors that might control disease expression in animals have been considered. It is known that factors such as breed, host susceptibility, environmental conditions, stress and pathogenicity of the particular BTV isolate influence BTV-host

interrelationship, and therefore, the ultimate disease expression (117, 118, 157). In addition, factors such as immunological modulation resulting from sequential infection by 2 or more different serotypes of the same virus, i.e., sequential heterotypic infections, must be considered as a possible basis of variation in desease expression. This mechanism has been proposed to explain the pathogenesis of other viral diseases including dengue hemorrhagic fever (DHF), an acute fever/shock syndrome affecting children and known to be associated with sequential dengue virus (DV) strain infections (60), and feline infectious peritonitis (FIP), the fatal systemic disease of cats thought to result from sequential infections with apparently different strains of FIP coronaviruses (171).

The results of previous studies suggest that the host's immune response might play a significant role in the development of lesions resulting from BTV infection. It has been demonstrated that 61.5% of the sheep having low neutralizing antibody titers to 1 of 2 BTV isolates developed clinically recognized bluetongue when challenged with either of the 2 isolates (19). The observation has also been made that the development of clinical signs and lesions in experimentally inoculated, susceptible sheep occurred several days after maximal virus infection of cells, and coincided with significant levels of detectable

serum antibody (148). Based on these observations, it is possible that clinically recognized disease only occurs as secondary infections in animals with pre-existing serum antibody to 1 or more of the serotypes of BTV.

Study of the immunologic responses of the host to sequential heterotypic BTV infections might provide a better understanding of the BTV-host interrelationship and facilitate the development of effective control measures. The purpose of this study was to evaluate the immunological response of mice to a second heterotypic inoculation of BTV, and to determine the pathogenetic significance of sequential viral exposure on the induction of virus-related lesions.

Specific experiments were designed to determine the immune response of adult mice to primary (single) BTV serotype infections (utilizing BTV 11), sequential homotypic, i.e., same serotype BTV infections, (utilizing BTV 11 for primary and secondary infections), and sequential heterotypic BTV infections (utilizing BTV 17 for primary and BTV 11 for secondary infections). Because it has previously been demonstrated that successive waves of enhancement (help) and suppression from thymus lymphocytes (T cells) occurred during the primary immunologic response to specific antigenic stimulation (143), the intervals between primary and secondary infections in the 2 experiments involving sequential infections were selected

to approximate those established times of maximal help and suppression.

A second group of experiments was designed to evaluate the effect of pre-existing passively acquired homotypic (utilizing anti-BTV 11 serum) or heterotypic (utilizing anti-BTV 17 serum) antibody (i.e., adoptive serum transfer immunization) on the immunologic response of adult mice to subsequent BTV 11 infection. A third group of experiments was designed to test the effect of adoptive spleen cell immunization, utilizing BTV 17-sensitized spleen cells, on the immunologic response of adult mice to subsequent heterotypic (BTV 11) infection. Sensitized spleen cells were collected from syngeneic donor mice at intervals representing optimal enhancing or suppressor activity (the same intervals selected for use in the first group of experiments).

All determinations of antibody responses in the present study were performed with the avidin/biotin enhanced-enzyme-linked immunosorbent assay (A/B-ELISA) as described in Chapter III. Assessment of tissues for the presence of BTV induced lesions was carried out on selected tissues with routine histologic and avidin/biotin-enhanced techniques as described in Chapter IV.

Materials and Methods

Experimental Animals

Adult C57Bl/6 mice (C57, parent stock obtained from the Jackson Laboratory, Bar Harbor, ME) were used in the present investigation. The mice were maintained on a standard laboratory chow as outlined by the National Research Council Guideline for Murine Nutrition, and were housed in a limited access, quarantined room until they were sacrificed for serum and tissue collection.

Mouse Adaptation of Virus

The 4 American serotypes of BTV [low passage isolates in Oxylated-Carbol-Glycerin solution (OCG)-treated sheep blood] were obtained from Dr. Stewart McConnell (Department of Veterinary Microbiology and Parasitology, Texas A & M University, College Station, TX) and adapted to suckling mice as previously described in Chapter III.

Serological Assay

Serum collected from each experimental group was assayed for BTV antibodies with the A/B-ELISA. The details of the assay and its procedure are presented in Chapter III. Attempts were made to test the serotype specificity of the antibody response of each group of mice by selected absorption of their sera with immunoadsorbents prepared

with BTV 11, BTV 13 and BTV 17 antigens.

Preparation of Immunoadsorbents

The procedure used to prepare the BTV immunoadsorbents was a modification of the well established glutaraldehyde cross-linking technique (7). Briefly, 18.0 ml of normal horse serum (NHS), serving as a protein expander, were mixed with 12.0 ml of soluble BTV antigen (stock BTV antigen prepared by fluorocarbon extraction as described in Chapter I) and the resultant solution was buffered with 4.0 ml of 0.2M phosphate buffered saline (PBS), pH 7.0. Approximately 1.5 ml of 25% aqueous glutaraldehyde were added dropwise as the solution was gently stirred. The insolubilized protein gel which formed approximately 15 minutes later, was allowed to stand at room temperature (RT) overnight before it was centrifuged at 1000 X g for 10 minutes. Any liquid present on the surface of the gel (usually less than 1.0 ml) was decanted. Excess glutaraldehyde was removed by repeated washing with dionized water (DH_2O). The gel was dispersed in 20 to 30 ml DH_2O and mixed at medium speed in an electric homogenizer (Virtis Research Equipment, Co., Gardiner, NY) for 10 to 20 seconds before it was centrifuged at 800 X g for 10 minutes at 4°C. The aqueous phase was discarded, and the wash procedure repeated twice more. Following the DH_2O washes, the gel was washed 3 times in similar manner

with 0.1M glycine-HCl buffer, pH 2.8. The mixture became granular after it was washed with glycine buffer. Three final washes with PBS were necessary before the immunoadsorbent was ready for use.

To employ the immunoadsorbent, appropriate volumes of diluted immune serum were mixed in centrifuge tubes with the insoluble protein. The mixture was gently agitated for 30 minutes at RT and allowed to stand for 4 hours at 4°C before it was centrifuged at 3000 X g for 10 minutes at 4°C. The aqueous phase was collected as the absorbed serum.

Light Microscopic Examination of Tissues

Experimental groups of mice were exsanguinated at designated times for serum and tissue collections. Representative tissues form various organ systems, i.e., the lymphoid (spleen and lymph node), integument (skin), urogenital (kidney and testicle/ovary), muscular (heart and skeletal muscle) and nervous (brain) systems and sections of liver were collected at the time of necropsy, fixed and processed by the acetone-paraffin method (30) described in Chapter II, and stained routinely with hematoxylin and eosin (H&E) (102) for the detection and evaluation of microscopic BTV-induced lesions.

Immunohistochemic Examination of Tissues

Paraffin embedded sections of liver, spleen, lymph nodes, and brain were collected and processed as described for the light microscopy studies. These sections were stained with the long-term incubation-avidin/biotin-peroxidase complex (ABC) staining technique described in Chapter II and illustrated in Fig. 19, p 83. Normal mouse tissues were stained and examined as negative controls.

Experimental Design

Part I: Comparison of primary (single) BTV infection with multiple homotypic and multiple heterotypic BTV infections. Thirty-nine adult C57 mice were separated into 3 major groups: A, B and C and further subdivided into numbered subgroups of 3 mice each as is shown in Tables 4, 5 and 6 respectively.

Group A mice received 0.5 ml of diluent administered by the intraperitoneal (IP) route on the same day that the primary infections of groups B and C were initiated. Subgroup A1 mice were killed on day 2 and served as uninfected controls. The A2, A3 and A4 subgroup mice were subsequently infected IP with BTV 11 [0.5 ml (4.45×10^4 MLD_{50}) of undiluted stock P_5 mouse brain suspension] on days 2, 7 and 16 respectively after the diluent injection. These 3 subgroups represented primary infections that controlled for any time sequence changes within the colony

TABLE 4. EXPERIMENTAL DESIGN FOR PRIMARY BLUETONGUE VIRUS (BTV) INFECTION: PART I, GROUP A.

Group	Subgroup	Treatment
A		Received primary BTV 11[a] infection after diluent injection on day 0 (time controls for colony)
	A1	Group control (non-infected), killed on day 2
	A2	Received primary infection on day 2
	A3	Received primary infection on day 7
	A4	Received primary infection on day 16

[a]BTV 11 = bluetongue virus serotype 11

TABLE 5. EXPERIMENTAL DESIGN FOR SEQUENTIAL HOMOTYPIC BLUETONGUE VIRUS (BTV) INFECTIONS: PART I, GROUP B.

Group	Subgroup	Treatment
B		Received secondary homotypic BTV 11[a] infection after primary BTV 11 infection on day 0
	B1	Group control (primary infection only), killed on day 2
	B2	Received secondary homotypic infection on day 2
	B3	Received secondary homotypic infection on day 7
	B4	Received secondary homotypic infection on day 16

[a]BTV 11 = bluetongue virus serotype 11

TABLE 6. EXPERIMENTAL DESIGN FOR SEQUENTIAL HETEROTYPIC BLUETONGUE VIRUS (BTV) INFECTIONS: PART I, GROUP C.

Group	Subgroup	Treatment
C		Received secondary heterotypic BTV 11[a] infection after primary BTV 17[b] infection on day 0
	C1	Group control (primary BTV 17 infection only), killed on day 2
	C2	Received secondary heterotypic infection on day 2
	C3	Received secondary heterotypic infection on day 7
	C4	Received secondary heterotypic infection on day 16
	C5	Received secondary homotypic BTV 17 infection on day 20 and tertiary heterotypic BTV 11 infection on day 24

[a]BTV 11 = bluetongue virus serotype 11

[b]BTV 17 = bluetongue virus serotype 17

and for any alterations to immunological response by additional inapparent infectious disease or other environmental changes that may have been present during the course of the experiment.

Each mouse in group B was initially infected (primary infection) with 0.5 ml of undiluted stock P_5/BTV 11 mouse brain suspension IP on day 0 (the same day that the group C mice were first infected). The mice in subgroup B1 were killed on day 2 and served as controls for the group. The B2, B3 and B4 subgroup mice were subsequently infected IP with 0.5 ml of undiluted stock P_5/BTV 11 mouse brain suspension on day 2 (subgroup B2), day 7 (subgroup B3) and day 16 (subgroup B4) to determine the effect of sequential homotypic BTV infections.

Each mouse in group C was infected IP with 0.5 ml (1.41×10^5 MLD_{50}) of undiluted stock P_5/BTV 17 mouse brain suspension as the primary infection on day 0. The mice in subgroup C1 were killed on day 2 and served as controls for the primary BTV 17 infection. Each mouse in subgroups C2, C3 and C4 was subsequently infected IP with 0.5 ML of undiluted stock P_5/BTV 11 mouse brain suspension on day 2 (subgroup C2), day 7 (subgroup C3) or day 16 (subgroup C4) to reflect the effect of sequential heterotypic BTV infections. Subgroup C5 mice received a second homotypic infection of P_5/BTV 17 mouse brain suspension on day 20 prior to a tertiary heterotypic infection of P_5/BTV 11

mouse brain suspension given on day 24.

The intervals chosen between the sequential infections described above were selected to reflect peak periods of enhancement or suppression following a primary immunization/infection. An early period of enhanced response (T cell helper activity) has been reported at 2 days after primary immunization with maximal enhancement reported to occur at approximately 16 days (143). Periods of suppressed response (T cell suppressor activity) were reported at 7 days after the primary immunization and at 4 days following a booster (secondary) immunization (143).

Fourteen days after the final infection, all mice in subgroups 2, 3 and 4 of groups A and B and subgroups 2, 3, 4 and 5 of group C were exsanguinated, and the sera collected from each subgroup pooled separately, aliquoted, and stored at -70°C until assayed for BTV antibody. Following exsanguination, the mice were necropsied, and specimens of selected organs collected and processed for histopathologic and immunohistochemic examination. Control subgroups were treated identically at 2 days after the injection of diluent (subgroup A1) or the primary infection with BTV 11 (subgroup B1) or BTV 17 (subgroup C1).

Part II: Effect of adoptive serum transfer immunization. The source of hyperimmune sera for this experiment was 15 adult C57 mice divided into 3 groups of 5 mice each. Two of the groups were hyperimmunized with BTV,

the first with stock P_5/BTV 11 mouse brain suspension and the second with stock P_5/BTV 17 mouse brain suspension. Three 0.5 ml, IP inoculations of the respective virus suspensions (4.5×10^4 MLD $_{50}$ of BTV 11 and 1.41×10^5 MLD $_{50}$ of BTV 17) were administered at 1 week intervals. The remaining group of mice, was injected with 0.5 ml of diluent IP, utilizing the same inoculation schedule and served as the source of control serum. Twenty-one days after the third injection, all mice were exsanguinated. Serum collected from each group was pooled separately, aliquoted, and stored at -70°C. At the time of use, representative aliquots from each of the 3 donor groups were rapidly thawed, diluted 1:10, filter sterilized, and each injected IP into 3 recipient groups of adult C57 mice at a rate of 0.5 ml per mouse. The recipient mice were designated as subgroups D1 (recipient of non-infected mouse serum), D2 (recipient of anti-BTV 11 serum) and D3 (recipient of anti-BTV 17 serum) in Table 7.

Twenty-four hours after serum administration, all mice were infected IP with 0.5 ml of thawed, undiluted stock P_5/BTV 11 mouse brain suspension. Fourteen days after infection, the mice were exsanguinated and necropsied as described in Part I above.

Part III: Effects of adoptive spleen cell transfer immunization. The source of BTV 17 sensitized-spleen cells

TABLE 7. EXPERIMENTAL DESIGN FOR ADOPTIVE IMMUNIZATION BY SERUM TRANSFER IMMEDIATELY BEFORE BLUETONGUE VIRUS (BTV) INFECTION: PART II, GROUP D.

Group	Subgroup	Treatment
D		Received immunization by adoptive serum transfer 24 hours before BTV 11[a] infection
	D1	Group control, recipient of non-infected mouse serum
	D2	Recipient of anti-BTV 11 serum
	D3	Recipient of anti-BTV 17[b] serum

[a]BTV 11 = bluetongue virus serotype 11

[b]BTV 17 = bluetongue virus serotype 17

was 20 adult C57 mice separated into 4 groups of 5 mice each and inoculated with a single 0.5 ml (1.41×10^5 MLD_{50}) IP injection of thawed, undiluted stock P_5/BTV 17 mouse brain suspension. At 2, 7 and 16 days post-inoculation (dpi), 1 group of the donor mice were exsanguinated and the spleens aseptically removed and diced into fine pieces in 15 ml of Puck's A saline (0.8% NaCl, 0.04% KCl, 0.1% glucose, 0.35% $NaHCO_3$, 0.002% phenol red in DH_2O), pH 7.8. After dissociation of cell clumps by pepetting, the supernatant (containing the BTV 17-sensitized spleen cells) was removed by pipette and centrifuged at 200 X g for 5 minutes. The aqueous phase was discarded, and the cell pellet resuspended in 4.0 ml of Puck's A saline before it was layered onto 4.0 ml of a sucrose polymer-diatrizoate density gradient (Ficol-Paque, Pharmacia Fine Chemical, Uppsala, Sweden) for the separation of erythroctyes and neutrophils from the spleen mononuclear leukocytes. The gradient was centrifuged at 400 X g for 25 minutes. After removing the top saline layer, the layer of splenic mononuclear leukocytes was pepetted into a centrifuge tube and resuspended in 5.0 ml of Puck's A saline. The cell suspension was centrifuged at 700 x g for 15 minutes, and the supernatant was discarded. Following 2 final washes (centrifugation at 500 X g for 15 minutes), the cell pellet was resuspended in 5.0 ml of Puck's A saline, and the cells counted in a hemocytometer. The percentage of viable cells

was assessed by the trypan blue dye exclusion test after which the cell suspension was centrifuged at 200 X g for 8 minutes. The cell pellet was resuspended in 1.0 ml of Puck's A saline, pH 7.0, and 0.2 ml of the suspension was injected intravenously (by way of the lateral tail vein) into each of a group of 5 normal adult C57 mice. At 20 dpi, the remaining group of 5 donor mice described above was reinoculated with a second IP dose (0.5 ml) of the thawed, undiluted stock P_5/BTV 17 mouse brain suspension (secondary infection). Four days following this secondary infection (day 24), the mice were killed and their spleens processed as described above. The sensitized spleen cells were injected intravenously into 5 normal adult C57 mice. Nonsensitized spleen cells (control cells) were obtained from 5 uninoculated adult C57 mice which were housed separately from the inoculated animals. These mice were killed and their spleens removed, processed as described above and the nonsensitized spleen cells were injected into 5 adult C57 mice designated as controls.

Following passive immunization by adoptive spleen cell transfer, the mice were maintained in groups of 5 as per the dpi of spleen cell harvest, i.e., control (subgroup E1), 2 dpi (subgroup E2), 7 dpi (subgroup E3), 16 dpi (subgroup E4 and 4 days post-secondary inoculation (subgroup E5), which are listed in Table 8. Twenty-four hours after adoptive immunization, all mice were infected

TABLE 8. EXPERIMENTAL DESIGN FOR ADOPTIVE SPLEEN CELL TRANSFER IMMUNIZATION IMMEDIATELY BEFORE BLUETONGUE VIRUS (BTV) INFECTION: PART III, GROUP E.

Group	Subgroup	Treatment
E		Immunization by adoptive spleen cell transfer (BTV 17[a]-sensitized cells) 24 hours before BTV 11[b] infection
	E1	Group control, received control spleen cells from non-infected donor mice
	E2	Recipient of spleen cells from donor mice infected with BTV 17 for 2 days
	E3	Recipient of spleen cells from donor mice infected with BTV 17 for 7 days
	E4	Recipient of spleen cells from donor mice infected with BTV 17 for 16 days
	E5	Recipient of spleen cells from donor mice primarily infected with BTV 17 for 20 days and secondarily infected BTV 17 for 4 days[c]

[a]BTV 17 = bluetongue virus serotype 17

[b]BTV 11 - bluetongue virus serotype 11

[c]2 separate BTV 17 infections were administered to the donor mice, the first on day 0 and the second on day 20. Spleen cells were harvested for transfer on day 24.

IP with 0.5 ml (4.45×10^4 MLD_{50}) of thawed, undiluted stock P_5/BTV 11 mouse brain suspension. All mice were exsanguinated 14 days post-infection as described in Part I above.

Results

Immunologic Responses

Antibody response to primary BTV infection (group A). Mice utilized for this experiment each received 0.5 ml of diluent on day 0, and were infected with 0.5 ml of undiluted stock P_5/BTV 11 mouse brain suspension on day 2, day 7 or day 16 (subgroups A2, A3 and A4 respectively, Table 4, p 98). Fourteen days post-infection each mouse was exsanguinated and necropsied for tissue harvest. Because each mouse received a single (primary BTV 11 infection (even though they were administered at different intervals after an injection of diluent) and was killed 14 days post-infection, the immune response to BTV in each subgroup would be expected to be the same. This hypothesis was confirmed when the sera were assayed for anti-BTV properties. Results demonstrated no significant differences in the absorbance values or the binding ratios (calculated by dividing the absorbance value of the test serum by the absorbance value of normal control serum included with each assay) of the 3 subgroups. Based on these results the data were combined and averaged to

represent a single subgroup (primary BTV 11 infection) designated as the A2, A3 and A4 average in Table 9.

Antibody response to sequential homotypic BTV infection (group B). The serum for each subgroup in group B was assayed against BTV 11, BTV 13 and BTV 17 antigens for the purpose of observing cross-reactivity of the various sera in this experiment and for the comparison with data obtained from the sequential heterotypic experiment (group C) below. The results are presented as binding ratios in Table 10. It was observed that all 3 of the BTV antigens were recognized by antibodies in each of the sera. However, larger binding ratios, reflecting higher antibody titers, were obtained from the assay of sera against BTV 11 antigen. It was also observed that sequential BTV 11 infections, even when measured as early as 2 days after the secondary infection (subgroup B2), resulted in a more substantial antibody response than did the primary BTV 11 infection (subgroups A2, A3 and A4 average). In addition, there was a slight decrease in the binding ratios calculated for the 7 dpi mice (subgroup B3) as compared to the 2 dpi mice (subgroup B2). This decrease was contrasted by a marked increase in the binding ratio for the 16 dpi mice (subgroup B4). This undulating antibody response very likely reflected the effects of sequential waves of T cell help and suppression on the production of antibody.

TABLE 9. ANTIBODY RESPONSE TO PRIMARY BLUETONGUE VIRUS SEROTYPE 11 (BTV 11) INFECTION: PART I, GROUP A.

Serum Designation or Subgroup	Treatment	Binding Ratio[a]
Normal Serum	Non-infected control serum	1.00
SRBC[b] Serum	Hyperimmunized anti-SRBC control serum	1.04
A1	Group A control, non-infected, killed on day 2 after injection of diluent on day 0	0.98
A2	Received primary BTV 11 infection on day 2 after injection of diluent on day 0	1.15
A3	Received primary BTV 11 infection on day 7 after injection of diluent on day 0	1.16
A4	Received primary BTV 11 infection on day 16 after injection of diluent on day 0	1.19
A2, A3 and A4 average $\pm$ standard deviation	Primary BTV 11 infection	1.17$\pm$ 0.02

[a] Binding Ratio = absorbance value of experimental serum divided by absorbance value of normal serum. Absorbance value of normal serum assayed with BTV 11 antigen was 0.408.

[b] SRBC = sheep red blood cell

TABLE 10. COMPARISON OF ANTIBODY RESPONSE IN PRIMARY BLUETONGUE VIRUS (BTV) INFECTION WITH SEQUENTIAL HOMOTYPIC AND SEQUENTIAL HETEROTYPIC BLUETONGUE VIRUS INFECTION

Subgroup	Treatment	Binding Ratios[a] for Antigen: BTV 11[b]	BTV 13[c]	BTV 17[d]
A1	Group A control (non-infected), killed on day 2 following injection of diluent on day 0	0.98	0.94	0.96
A2, A3 and A4 average	Primary BTV 11 infection	1.17	1.01	1.00
B1	Group B control (primary BTV 11 infection only), killed on day 2	1.14	0.97	0.98
B2	Received secondary homotypic BTV 11 infection on day 2 after primary BTV 11 infection on day 0	1.42	1.18	1.20
B3	Received secondary homotypic BTV 11 infection on day 7 after primary BTV 11 infection on day 0	1.32	1.06	1.08
B4	Received secondary homotypic BTV 11 infection on day 16 after primary BTV 11 infection on day 0	1.95	1.71	1.66
C1	Group C control (primary BTV 17 infection only), killed on day 0	1.05	1.01	0.99

TABLE 10 Continued

Subgroup	Treatment	Binding Ratios[a] for Antigen: BTV 11[b]	BTV 13[c]	BTV 17[d]
C2	Received secondary heterotypic BTV 11 infection on day 2 after primary BTV 17 infection on day 0	1.60	1.38	1.39
C3	Received secondary heterotypic BTV 11 infection on day 7 after primary BTV 17 infection on day 0	1.58	1.33	1.69
C4	Received secondary heterotypic BTV 11 infection on day 16 after primary BTV 17 infection on day 0	2.06	1.57	1.69
C5	Received homotypic BTV 17 infection on day 20 and tertiary heterotypic BTV 11 infection on day 24 after primary BTV 17 infection on day 0	3.36	2.68	3.17

[a]Binding Ratios = absorbance value of experimental serum divided by absorbance value of normal serum. Absorbance value of normal serum assayed with BTV 11 antigen was 0.408, with BTV 13 it was 0.380 and with BTV 17 it was 0.296. Absorbance read at 490 mn.

[b]BTV 11 = bluetongue virus serotype 11

[c]BTV 13 = bluetongue virus serotype 13

[d]BTV 17 = bluetongue virus serotype 17

Antibody response to sequential heterotypic BTV infections (group C). As with the sequential homotypic infection data, there was a substantial increase in the measurable anti-BTV activity in the serum from each of the treatment subgroups (subgroups C1, C2, C3, C4 and C5 in Table 10, p. 111) as compared to the primary BTV 11 infection data. Once again, the larger binding ratios, reflecting higher antibody titers, were calculated when the sera were tested against BTV 11 antigen , even though all 3 antigens were recognized by the antibody activity in the sera. A similar undulating trend was noted between the 2 dpi (subgroup C2), the 7 dpi (subgroup C3) and the 16 dpi (subgroup C4) subgroups, as was between the corresponding subgroups (subgroups B2, B3 and B4) in the sequential homotypic experiment. The exaggerated response measured in subgroup C5 mice (those mice which received a primary and a secondary BTV 17 infection prior to the tertiary BTV 11 infection) was interesting. The binding ratios for this group were greater than 1.6 times the binding ratios for subgroup C4 mice (those mice which received a primary BTV 17 infection followed 16 days later by a secondary BTV 11 infection). This observation suggests that the secondary BTV 17 infection administered to subgroup C5 mice resulted in an amnestic response in those mice. However, the ultimate effect (i.e., enhancement or suppression of the antibody response) due to the tertiary infection could not

be fully evaluated because the effects of a homotypic tertiary BTV 17 infection were not investigated in the present study.

Effect of adoptive immunization by serum transfer (group D). The binding ratios for the 3 subgroups of mice treated with immune or normal serum are listed in Table 11. Administration of normal mouse serum to the control mice (subgroup D1) prior to BTV 11 infection resulted in a binding ratio intermediate between those for the treatment subgroups (subgroups D2 and D3). There was no evidence of an active antibody response in mice given the homologous antibody (anti-BTV 11) prior to BTV 11 infection (subgroup D2). However, the binding ratio for mice given antibody against the heterotypic BTV serotype (BTV 17) prior to BTV 11 infection (subgroup D3) was greater than that of the control animals but was essentially the same as that for mice not passively immunized before BTV 11 infection (subgroups A2, A3 and A4 average). These results suggested that the pre-existence of homologous antibody neutralized or aided in the elimination of the subsequent virus inoculum prior to induction of an immunologic response. In contrast, the pre-existence of heterologous antibody appeared not to neutralize or eliminate the subsequent virus inoculum and may have enhanced the antibody response to the subsequent virus infection.

TABLE 11. EFFECT OF ADOPTIVE IMMUNIZATION BY SERUM TRANSFER IMMEDIATELY BEFORE BLUETONGUE VIRUS INFECTION (GROUP D).

AND

COMPARISON WITH THE ANTIBODY RESPONSE TO PRIMARY BLUETONGUE VIRUS INFECTION

Subgroup	Treatment	Binding Ratio[a]
D1	Group D control, received non-infected serum 24 hours before BTV 11[b] infection	1.08
D2	Received anti-BTV 11 serum 24 hours before BTV 11 infection	1.01
D3	Received anti-BTV 17[c] serum 24 hours before BTV 11 infection	1.18
A2, A3 and A4 average	Primary BTV 11 infection	1.17

[a] Binding Ratio = absorbance value of experimental serum divided by absorbance value of normal serum. Absorbance value of normal serum assayed with BTV 11 antigen was 0.408. Absorbance read at 490 nm.

[b] BTV 11 = bluetongue virus serotype 11

[c] BTV 17 = bluetongue virus serotype 17

Effect of adoptive immunization by transfer of sensitized spleen cells (group E). Binding ratios for the sera of the 5 subgroups of mice receiving sensitized or normal spleen cells 24 hours before infection are listed in Table 12. The adoptive transfer of either normal (subgroup E1) or 2, 7 or 16 day-post BTV 17-sensitized spleen cells (subgroups E2, E3 and E4 respectively) had little measurable effect on the antibody response (represented by the binding ratios which approximated 1.00) of recipient mice to subsequent BTV 11 infection. The adoptive transfer of spleen cells from mice given 2 injections of BTV 17 virus prior to being given an infection with BTV 11 (subgroup E5) resulted in only minimal increase in the binding ratio when assayed with BTV 11 antigen. These results tend to suggest that the adoptive transfer of BTV-sensitized spleen cells affected some type of suppression (either specific or nonspecific) on the immune response to subsequent BTV 11 infection. Although binding ratios of less than 1.00 observed when the sera were assayed with BTV 13 and BTV 17 antigens suggested the possibility of maximal suppression of group specific BTV antibody response by this type of immunization procedure, the values observed were not statistically different from 1.00.

TABLE 12. EFFECT OF ADOPTIVE IMMUNIZATION BY SENSITIZED SPLEEN CELL TRANSFER IMMEDIATELY BEFORE BLUETONGUE VIRUS (BTV) INFECTION (GROUP E)

AND

COMPARISON WITH THE ANTIBODY RESPONSE TO PRIMARY BLUETONGUE VIRUS INFECTION

Subgroup	Treatment	Binding Ratios[a] for Antigen: BTV 11[b]	BTV 13[c]	BTV 17[d]
E1	Group E control, received control spleen cells from non-infected donor mice. BTV 11 infection given 24 hours later	1.03	0.88	0.99
E2	Received sensitized spleen cells from donor mice infected with BTV 17 for 2 days. BTV 11 infection given 24 hours later	0.99	0.94	0.96
E4	Received sensitized spleen cells from donor mice infected with BTV 17 for 7 days. BTV 11 infection given 24 hours later	1.03	0.96	0.99
E4	Received sensitized spleen cells from donor mice infected with BTV 17 for 16 days. BTV 11 infection given 24 hours later	1.02	0.96	0.94

TABLE 12 Continued

Subgroup	Treatment	Binding Ratios[a] for Antigen: BTV 11[b]	BTV 13[c]	BTV 17[d]
E5	Received sensitized spleen cells from donor mice primarily infected with BTV 17 for 20 days and secondarily infected with BTV 17 for 4 days.[e] BTV 11 infection given 24 hours later	1.07	0.96	0.95
A2, A3 and A4 average	Primary BTV 11 infection	1.17	0.96	1.00

[a]Binding Ratio = absorbance value of experimental serum divided by absorbance value of normal serum. Absorbance value of normal serum assayed with BTV 11 antigen was 0.408, with BTV 13 antigen it was 0.380 and with BTV 17 antigen it was 0.296. Absorbance read at 490 nm.

[b]BTV 11 = bluetongue virus serotype 11

[c]BTV 13 = bluetongue virus serotype 13

[d]BTV 17 = bluetongue virus serotype 17

[e]2 separate BTV 17 infections were administered to the donor mice, the first on day 0 and the second on day 20. Spleen cells were harvested for transfer on day 24.

Serotype-specificity of antibody response. Sera from all of the experimental groups in the present study were absorbed sequentially with BTV 13 and BTV 17 immunoadsorbents. The BTV 13 immunoadsorbent was employed to remove BTV group-specific antibodies from the sera and the BTV 17 immunoadsorbent was employed to remove BTV 17 type-specific as well as any remaining group-specific antibodies from the sera. Sequential absorption of the sera with the 2 immunoadsorbents was utilized in an attempt to remove all BTV antibody from the sera except that antibody which was type-specific for BTV 11. Results of the absorption experiments are presented as binding ratios in Tables 13, 14 and 15. The observation that absorption of the normal control serum and the mouse anti-sheep red blood cell (SRBC) serum with BTV 13 and BTV 17 antigens substantially reduced (approximately 50%) the absorbance value of these 2 sera was noteworthy. Possible explanations for the reduced absorbance values by normal sera are: (1) antibody in the normal sera which cross-reacted with the BTV antigen, (2) non-immunoglobulin proteins of normal sera adhering to the plate, or (3) the antigen coated plate was activating complement components. For possibilities 2 or 3 to affect the absorbance values, the immunoreagents used in subsequent steps would have to be reacting with the indicated proteins.

TABLE 13. SEROTYPE-SPECIFICITY OF ANTIBODY RESPONSE: SUMMARY OF BINDING RATIOS[a] FOR NON-ABSORBED AND ABSORBED[b] SERA FOR ALL EXPERIMENTAL GROUPS ASSAYED WITH BLUETONGUE VIRUS SEROTYPE 11 (BTV 11).

Serum Designation or subgroup	Treatment	BTV 11 Binding Ratios Non-absorbed	Absorbed
Normal Serum	Non-infected control serum	1.00	1.00
SRBC[c] Serum	Hyperimmunized anti-SRBC control serum	1.04	1.03
A1	Group A control (non-infected), killed on day 2 after injection of diluent on day 0	0.98	1.02
A2, A3 and A4 average	Primary BTV 11 infection	1.17	1.25
B1	Group B control (primary BTV 11 infection only) killed on day 2 after BTV 11 infection on day 0	1.14	1.21
B2	Received secondary homotypic BTV 11 infection on day 2 after primary BTV 11 infection on day 0	1.42	1.43
B3	Received secondary homotypic BTV 11 infection on day 7 after primary BTV 11 infection on day 0	1.32	1.30
B4	Received secondary homotypic BTV 11 infection on day 16 after primary BTV 11 infection of day 0	1.95	1.56

TABLE 13 Continued

Serum Designation or subgroup	Treatment	BTV 11 Binding Ratios Non-absorbed	Absorbed
C1	Group C control (primary BTV 17 infection only), killed on day 2 after BTV 17 infection on day 0	1.05	1.19
C2	Received secondary heterotypic BTV 11 infection on day 2 after primary BTV 17 infection on day 0	1.60	1.73
C3	Received secondary heterotypic BTV 11 infection on day 7 after primary BTV 17 infection on day 0	1.58	1.81
C4	Received secondary heterotypic BTV 11 infection on day 16 after primary BTV 17 infection on day 0	2.06	1.99
C5	Received secondary homotypic BTV 17 infection on day 20 and tertiary heterotypic BTV 11 infection on day 24 after primary BTV 17 infection on day 0	3.36	2.10
D1	Group D control, received non-infected mouse serum 24 hours before BTV 11 infection	1.08	1.58
D2	Received anti-BTV 11 serum 24 hours before BTV 11 infection	1.01	1.56
D3	Received anti-BTV 17 serum 24 hours before BTV 11 infection	1.18	1.52
E1	Group E control, received control spleen cells from non-infected donor mice. BTV 11 infection given 24 hours later	1.03	1.62

TABLE 13 Continued

Serum Designation or subgroup	Treatment	BTV 11 Binding Ratios Non-absorbed	Absorbed
E2	Received sensitized spleen cells from donor mice infected with BTV 17 for 2 days. BTV 11 infection given 24 hours later	0.99	1.54
E3	Received sensitized spleen cells from donor mice infected with BTV 17 for 7 days. BTV 11 infection given 24 hours later	1.03	1.56
E4	Received sensitized spleen cells from donor mice infected with BTV 17 for 16 days. BTV 11 infection given 24 hours later	1.02	1.64
E5	Received sensitized spleen cells from donor mice primarily infected with BTV 17 for 20 days and secondarily infected with BTV 17 for 4 days. BTV 11 infection given 24 hours later	1.07	1.72

[a]Binding Ratios = absorbance value of experimental serum divided by absorbance value of normal serum. Absorbance value of normal serum assayed with BTV 11 antigen was 0.408. Absorbance read at 490 nm.

[b]Absorbed sera = serum absorbed first with BTV 13 immunoadsorbent and second with BTV 17 immunoadsorbent. Absorbance value of absorbed normal serum assayed with BTV antigen was 0.191.

[c]SRBC = sheep red blood cell

TABLE 14. SEROTYPE-SPECIFICITY OF ANTIBODY RESPONSE: SUMMARY OF BINDING RATIOS[a] FOR NON-ABSORBED AND ABSORBED[b] SERA FOR ALL EXPERIMENTAL GROUPS ASSAYED WITH BLUETONGUE VIRUS SEROTYPE 13 (BTV 13).

Serum Designation or subgroup	Treatment	BTV 13 Binding Ratios Non-absorbed	Absorbed
Normal Serum	Non-infected control serum	1.00	1.00
SRBC[c] Serum	Hyperimmunized anti-SRBC control serum	1.02	0.94
A1	Group A control (non-infected), killed on day 2 after injection of diluent on day 0	0.94	1.01
A2, A3 and A4 average	Primary BTV 11 infection	1.01	1.11
B1	Group B control (primary BTV 11 infection only) killed on day 2 after BTV 11 infection on day 0	0.97	1.08
B2	Received secondary homotypic BTV 11 infection on day 2 after primary BTV 11 infection on day 0	1.18	1.05
B3	Received secondary homotypic BTV 11 infection on day 7 after primary BTV 11 infection on day 0	1.06	1.00
B4	Received secondary homotypic BTV 11 infection on day 16 after primary BTV 11 infection of day 0	1.71	1.28

TABLE 14 Continued

Serum Designation or subgroup	Treatment	BTV 13 Binding Ratios Non-absorbed	Absorbed
C1	Group C control (primary BTV 17 infection only), killed on day 2 after BTV 17 infection on day 0	1.01	1.02
C2	Received secondary heterotypic BTV 11 infection on day 2 after primary BTV 17 infection on day 0	1.38	1.12
C3	Received secondary heterotypic BTV 11 infection on day 7 after primary BTV 17 infection on day 0	1.33	1.15
C4	Received secondary heterotypic BTV 11 infection on day 16 after primary BTV 17 infection on day 0	1.57	1.22
C5	Received secondary homotypic BTV 17 infection on day 20 and tertiary heterotypic BTV 11 infection on day 24 after primary BTV 17 infection on day 0	2.68	1.42
D1	Group D control, received non-infected mouse serum 24 hours before BTV 11 infection	0.98	0.98
D2	Received anti-BTV 11 serum 24 hours before BTV 11 infection	1.03	1.05
D3	Received anti-BTV 17 serum 24 hours before BTV 11 infection	1.03	1.19
E1	Group E control, received control spleen cells from non-infected donor mice. BTV 11 infection given 24 hours later	0.88	1.01

TABLE 14 Continued

Serum Designation or subgroup	Treatment	BTV 13 Binding Ratios Non-absorbed	Absorbed
E2	Received sensitized spleen cells from donor mice infected with BTV 17 for 2 days. BTV 11 infection given 24 hours later	0.94	1.06
E3	Received sensitized spleen cells from donor mice infected with BTV 17 for 7 days. BTV 11 infection given 24 hours later	0.96	1.01
E4	Received sensitized spleen cells from donor mice infected with BTV 17 for 16 days. BTV 11 infection given 24 hours later	0.96	1.03
E5	Received sensitized spleen cells from donor mice primarily infected with BTV 17 for 20 days and secondarily infected with BTV 17 for 4 days. BTV 11 infection given 24 hours later	0.99	1.21

[a]Binding Ratios = absorbance value of experimental serum divided by absorbance value of normal serum. Absorbance value of normal serum assayed with BTV 13 antigen was 0.380. Absorbance read at 490 nm.

[b]Absorbed sera = serum absorbed first with BTV 13 immunoadsorbent and second with BTV 17 immunoadsorbent. Absorbance value of absorbed normal serum assayed with BTV antigen was 0.276.

[c]SRBC = sheep red blood cell

TABLE 15. SEROTYPE-SPECIFICITY OF ANTIBODY RESPONSE: SUMMARY OF BINDING RATIOS[a] FOR NON-ABSORBED AND ABSORBED[b] SERA FOR ALL EXPERIMENTAL GROUPS ASSAYED WITH BLUETONGUE VIRUS SEROTYPE 17 (BTV 17).

Serum Designation or subgroup	Treatment	BTV 17 Binding Ratios Non-absorbed	Absorbed
Normal Serum	Non-infected control serum	1.00	1.00
SRBC[c] Serum	Hyperimmunized anti-SRBC control serum	0.97	1.04
A1	Group A control (non-infected), killed on day 2 after injection of diluent on day 0	0.96	0.99
A2, A3 and A4 average	Primary BTV 11 infection	1.00	1.05
B1	Group B control (primary BTV 11 infection only) killed on day 2 after BTV 11 infection on day 0	0.98	1.08
B2	Received secondary homotypic BTV 11 infection on day 2 after primary BTV 11 infection on day 0	1.20	1.54
B3	Received secondary homotypic BTV 11 infection on day 7 after primary BTV 11 infection on day 0	1.08	1.06
B4	Received secondary homotypic BTV 11 infection on day 16 after primary BTV 11 infection of day 0	1.66	1.39

TABLE 15 Continued

Serum Designation or subgroup	Treatment	BTV 17 Binding Ratios Non-absorbed	Absorbed
C1	Group C control (primary BTV 17 infection only), killed on day 2 after BTV 17 infection on day 0	0.99	1.04
C2	Received secondary heterotypic BTV 11 infection on day 2 after primary BTV 17 infection on day 0	1.39	1.21
C3	Received secondary heterotypic BTV 11 infection on day 7 after primary BTV 17 infection on day 0	1.28	1.16
C4	Received secondary heterotypic BTV 11 infection on day 16 after primary BTV 17 infection on day 0	1.69	1.38
C5	Received secondary homotypic BTV 17 infection on day 20 and tertiary heterotypic BTV 11 infection on day 24 after primary BTV 17 infection on day 0	3.17	1.54
D1	Group D control, received non-infected mouse serum 24 hours before BTV 11 infection	0.91	1.16
D2	Received anti-BTV 11 serum 24 hours before BTV 11 infection	0.97	0.95
D3	Received anti-BTV 17 serum 24 hours before BTV 11 infection	0.99	1.11
E1	Group E control, received control spleen cells from non-infected donor mice. BTV 11 infection given 24 hours later	0.99	1.02

TABLE 15 Continued

Serum Designation or subgroup	Treatment	BTV 17 Binding Ratios Non-absorbed	Absorbed
E2	Received sensitized spleen cells from donor mice infected with BTV 17 for 2 days. BTV 11 infection given 24 hours later	0.96	1.12
E3	Received sensitized spleen cells from donor mice infected with BTV 17 for 7 days. BTV 11 infection given 24 hours later	0.99	1.06
E4	Received sensitized spleen cells from donor mice infected with BTV 17 for 16 days. BTV 11 infection given 24 hours later	0.94	1.10
E5	Received sensitized spleen cells from donor mice primarily infected with BTV 17 for 20 days and secondarily infected with BTV 17 for 4 days. BTV 11 infection given 24 hours later	0.95	1.17

[a]Binding Ratios = absorbance value of experimental serum divided by absorbance value of normal serum. Absorbance value of normal serum assayed with BTV 17 antigen was 0.296. Absorbance read at 490 nm.

[b]Absorbed sera = serum absorbed first with BTV 13 immunoadsorbent and second with BTV 17 immunoadsorbent. Absorbance value of absorbed normal serum assayed with BTV antigen was 0.193.

[c]SRBC = sheep red blood cell

The most critical observation to the absorption study is that even with this sequential 2 step procedure which dramatically reduced the absorbance values for all sera, there was still antibody activity remaining in the absorbed sera for group specific BTV antigens. This was indicated by the high binding ratios for mice in subgroups B2 (assayed with the BTV 17 antigen) and B4 (assayed with both the BTV 17 and BTV 13 antigens). The central question concerning the effect of a sequential heterotypic infection versus a homotypic sequence on the production of antibody to serotype-specific determinants, (i.e., BTV 11), could not be quantitated because of the BTV group-specific antibody left in the serum samples. Although conclusions could not be made quantitatively, an observation contrary to the older beliefs about response to different BTV serotypes (based on in vitro serum neutralization tests) could be made. The previously held view reasoned that because serotypes could be clearly differentiated and were usually not cross-reactive by in vitro serum neutralization tests, infection of an animal by 1 serotype would not alter the host response when infection by a different serotype occurred. In contrast to the predictions made with this view, it was clear that the absorbed serum binding ratios of sequential heterotypically infected mice (subgroups C2, C3 and C4 assayed with BTV 11 antigen) were significantly higher than either the absorbed serum binding ratios for

the primarily (subgroups A2, A3 and A4 average) or the sequential homotypically BTV 11 infected mice (subgroups B2, B3 and B4).

Absorption of the sera also generally increased the sensitivity of the A/B-ELISA for low serological response. In particular, the binding ratios calculated for the groups of the 2 passive immunization experiments (groups D and E) were larger for the absorbed than for the corresponding non-absorbed sera. A final observation was that the binding ratios for the sera from all the tests groups were substantially higher when assayed with BTV 11 antigen following immunoabsorption. This finding suggested that most of the antiviral activity in the sera was directed against BTV 11, the virus serotype used as the secondary infection in all sequential exposure experiments.

Evaluation of Virus-Induced Lesions

Clinical signs of illness were not observed in any of the adult mice infected IP with BTV mouse brain suspensions. Routine histopathologic examination (H & E staining) of the spleen, liver, mesenteric lymph node, skin, kidney, ovary, testicle, heart, skeletal muscle, and brain revealed no significant deviations from normal at the 14 day post-infection interval. Neither was there any specific staining observed in the paraffin embedded sections of brain, liver, spleen, or mesenteric lymph node taken from

those same mice and examined with the modified ABC immunoperoxidase staining technique. Specific staining was observed however, in all of the positive control sections, i.e., BTV infected suckling mouse brain, assayed simultaneously with the experimental sections, using identical reagents.

It was recently reported that mouse-adapted BTV could be recovered from the heart and spleen of mice following IP inoculation for up to 8 days (82). Based on that study, absence of specific staining of the experimental tissues in the present study could be anticipated as the test animals were killed for tissue processing 14 days after the last BTV inoculation. As a means of investigating the problem, 7 adult C57 mice were inoculated IP with 0.5 ml (4.45×10^4 MLD_{50}) of undiluted stock P_5/BTV 11 mouse brain suspension. Each consecutive day post-inoculation, 1 mouse was killed for tissue harvest until all 7 were killed. Sections of heart, spleen, liver, and mesenteric lymph node were processed, embedded, and stained in a like manner as were the test tissues. Specific staining was observed in the heart (endothelial cells) and spleen (large mononuclear leukocytes) of the mice killed on dpi 5 and 6 (Fig. 20 and 21). Specific staining was not observed in the lung or liver of these mice, nor was it observed in any of the tissues of mice killed on the other days.

Fig. 20. Positive immunoperoxidase staining of myocardial endothelial cells (arrows) in adult mouse 5 days post-intraperitoneal bluetongue virus (BTV) inoculation. Long-term incubation-avidin/biotin-peroxidase complex (ABC) method of staining, 760X.

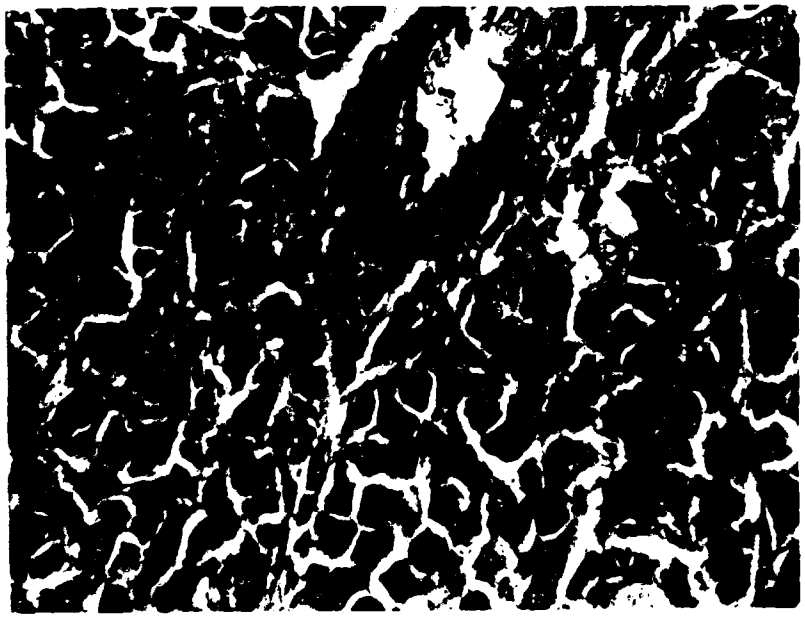

Fig. 21. Positive immunoperoxidase staining in the cytoplasm of large mononuclear leukocytes (arrows) in the spleen of an adult mouse 6 days after intraperitoneal inoculation with bluetongue virus (BTV). Hemosiderin (H) granules are also present in the field. Long-term incubation- avidin/biotin-peroxidase complex (ABC) method of staining, 640X.

Discussion

The present study was designed to study the immunologic and pathologic consequences of sequential heterotypic BTV infections in mice. In so doing, the effects of primary and sequential homotypic BTV infections also had to be evaluated. Results of the immunologic investigations indicated that the adult C57 mice utilized in the study did not respond well immunologically to primary infection with P_5/BTV 11 mouse brain suspension. This observation was puzzling in that previous reports indicated that mice responded well immunologically to BTV infection (81, 82, 119). However, those previous studies utilized different strains of mice and different assay systems. Also, in the present study the date of serum harvest for the assay was 14 dpi, a time when maximal T cell help and, therefore, maximal antibody response was expected to have been induced in the mice. A possible explanation for the low primary response in these mice to primary BTV infection is the fact that the mice were injected with diluent prior to BTV 11 inoculation. This seemingly insignificant challenge to the animals' immune system may have resulted in an undetermined nonspecific immune suppression, which resulted in a lack of response to the virus. A more feasible explanation is that the C57 strain of mice is genetically a low responder strain to all bluetongue viruses, or more specifically to the particular

BTV 11 serotype used in the present study. The response to BTV might also be dose dependent, and the infecting dose may have been too small or large to induce a sufficient immunologic response.

Whatever the reason for the minimal response to primary BTV 11 infection, the problem was not manifested in the immunologic response of mice sequentially infected with homotypic or heterotypic BTV serotypes. The immune response to both types of infection were substantially increased over that of the single BTV 11 infection. These results are in agreement with those of another study in which a substantial increase was noted in the anti-BTV activity of sera obtained from mice which received 2 separate inoculations of BTV as opposed to a single inoculation (81). Interestingly, the course of the response correlated well with the previously reported scheme of successive T cell help and suppression during the primary immunologic response to antigenic stimulation (143). This observation might become important if this scheme were to hold true for similar infections in ruminants. Should an animal be sequentially infected with BTV during the maximal suppression phase of the T cell regulation scheme, a "super" infection could occur, whereby the virus would be permitted to become widely disseminated throughout the animal's body. In contrast, if the secondary infection occurred during the maximal help phase of the T cell

regulation, and if the enhanced antibody response was helpful, elimination of the secondary infection would be expected and the animal should recover uneventfully. However, if the enhanced immunologic response induced an immunopathologic event, as occurs with DHF (58, 59) and is proposed for FIP (172), the possibility of subsequent clinical disease would be greatly increased.

The data demonstrates that the antibody response to sequential heterotypic BTV infections, i.e., BTV 17 followed by BTV 11, is greater than that to sequential homotypic BTV 11 infections. However, in order to better evaluate this data, a group of mice should have been infected sequentially with BTV 17 virus. As this was not included in the design of the experiment, any conclusions drawn from the present experiments must be limited to observations of sequential BTV 11 infections as opposed to infections of BTV 17 followed sequentially by BTV 11. Here again, this apparent enhancement of the antibody response might cause varying consequences as discussed above in ruminant species, if the same phenomenon holds true across species lines.

The present experiments with adoptive transfer of anti-BTV sera indicated that pre-existence of homotypic BTV antibody was either effectual in the neutralization of the subsequent virus infection, thereby arresting BTV antibody production; or in some way modulated the immune system so

that antibody was not produced to the subsequent viral infection or that antibody which was produced was not detected by the assay method employed. However, the results suggested that pre-existence of heterotypic BTV antibody may have enhanced, rather than neutralized, the subsequent infection. Should an enhanced immunologic response occur, as suggested by this latter observation, there would be an opportunity for the precipitation of an immunopathologic event such as occurs with DHF (58, 59) and is proposed for FIP (172).

Adoptive transfer of BTV sensitized spleen cells could have resulted in marked elaboration of antibody as was observed with Coxsackie B4 virus (28). However, in the present study, antibody against BTV was not detected in the sera of recipient mice after the transfer of sensitized spleen cells. Similar results have been observed with adoptive transfer of DV sensitized spleen cells followed by viral challenge. Those results led the investigators to conclude that cell-mediated immunity apparently had no protective role in DV infections (27). Such might not be the case with BTV infections, as recent publications indicate that BTV infection in mice induced the production of cytotoxic T cells (CTL) which show virus and H-2 restriction (81, 82). It has also been demonstrated that CTL have the ability to destroy virus-infected cells early in infection, and therefore, may prevent viral spread (175).

If CTL were produced during the BTV sensitizing process in the donor mice, then it seems likely that following transfer of the cells (including CTL) to recipient mice, that they, along with the host's defense mechanisms, could eliminate the virus before sufficient viral antigen was produced by cells and before the production of anti-BTV antibody was induced. It is also possible that a feedback suppression of antibody formation was initiated by those T cells adoptively transferred to the recipient mice which resulted in inhibited of anti-BTV antibody production until such time as the virus was eliminated by other means. Such a suppression mechanism has been proposed for adoptive spleen cell immunization of mice against ectromelia virus (126).

Results of the absorption study were encouraging. It was evident that the absorbed serum binding ratios of sequential heterotypically infected mice were significantly higher than either the absorbed serum binding ratios for the primarily or the sequential homotypically infected mice. In addition, results of the procedure suggested that most of the antiviral activity observed in the sera of sequential heterotypically infected mice was directed against the secondarily infecting serotype (BTV 11). Although quantitative conclusions cannot be drawn from the data, it appeared that although the A/B-ELISA was a group-specific assay; modifications such as the selective

absorption of sera might be useful in the adaptation of the assay to type-specific uses should satisfactory, purified type-specific antigens become available.

As was concluded in previous studies (81, 82, 107, 119), the results of the present study indicated that the mouse-BTV infection model was not an effective model system for the study of typical ruminant bluetongue disease. None of the adult mice infected, or otherwise exposed to BTV, became clinically ill. Therefore, it was not surprising when histopathologic and immunohistochemical examination of various tissues from the mice failed to demonsatrate lesions or specific staining, respectively. The usefulness of the modified ABC immunoperoxidase staining technique for the detection of BTV antigen in tissue was however, demonstrated by this study. Bluetongue virus antigen was readily detected in sections of infected suckling mouse brain (positive controls) and in the hearts and spleens of adult mice 5 and 6 days post-IP inoculation with P_5/BTV 11 mouse brain suspension. This technique should lend itself well to the examination of sheep tissues simply by substituting a biotinylated anti-sheep IgG reagent in place of the anti-mouse IgG reagent used in the present study.

CHAPTER VI

SUMMARY

The immunologic response to sequential heterotypic bluetongue virus (BTV) infections was studied in adult C57Bl/6 mice. For the purpose of this study, an avidin/biotin-enhanced enzyme-linked immunoabsorbent assay (A/B-ELISA) and an avidin/biotin-enhanced immunoperoxidase staining technique were modified for use with the BTV-mouse model system. Both of these assays make use of the newly developed avidin-biotin-peroxidase complex (ABC) which is based on the extraordinarily high binding affinity of avidin for biotin (dissociation constant of 10^{-15} M). Avidin is a 68,000 molecular weight glycoprotein present in egg white which has 4 binding sites for biotin, a small molecular weight vitamin.

The A/B-ELISA system was obtained as a commercial ABC kit. However, before the kit could be adapted as an assay of BTV antibody in sera of experimentally inoculated mice, several variables involved in the procedure had to be investigated so that the maximum potential of the assay was achieved. The variables investigated include:

1. Antigen coating buffers used to adsorb the antigen to the test plate
2. Two methods of antigen preparation
3. Antigen and antisera dilutions
4. Diluents for multiple reagents
5. Substrate solutions
6. Microtiter plates
7. Rinsing procedures

The titration of antibody by the A/B-ELISA method was based on quantitating the amount of enzyme generated product spectrophotometrically. The results were expressed as a binding ratio which was calculated by dividing the absorbance values of the dilutions of the various positive sera by the absorbance values of the corresponding dilutions of normal mouse sera. This binding ratio standardizes results accommodating for variation in trials run on different days. Analyzing the data based on the binding ratios, the best results were obtained with a combination of:

1. Coating buffer: carbonate buffer (pH 9.6)
2. Antigen: fluorocarbon extract of BTV infected suckling mouse brain diluted 1:200
3. Primary antiserum: Mouse anti-BTV diluted 1:100
4. Reagent diluent: 0.01M phosphate buffered saline (pH 7.6) containing 0.1% Tween 20, 1.0% bovine serum albumin and 0.05% gelatin (PTBG)
5. Substrate solution: equal parts of 0.08% orthophenylenediamine dihydrochloride (OPD) in 0.1M citrate buffer (pH 5.0) and 0.03% hydrogen peroxide (H_2O_2); 4 normal sulfuric acid (4N H_2SO_4) was used to stop the reaction at 30 minutes and absorbance was read at 490 nm
6. Microtiter plate: Immunolon II, Dynatech Laboratories, Inc.
7. Rinsing procedure: 7 rinses per washing step except after enzyme conjugate step; rinse fluid consisted of 0.15M sodium chloride (NaCl) solution containing 0.5% Tween 80

With these test procedures, it was demonstrated that the A/B-ELISA was an effective assay for the detection of serum antibody produced against BTV in the mouse.

The avidin/biotin-enhanced immunoperoxidase tissue staining system was obtained as a commercial ABC kit. The

recommendations supplied by the manufacturer failed to adequately demonstrate BTV antigen in previously inoculated mouse brain tissue. A series of modifications of the staining procedure greatly improved the sensitivity of the technique. The modifications included:

1. Acetone dehydration-low melting point (50-53°C) paraffin embedding process for formalin fixed tissues
2. A long-term incubation procedure which entailed the immersion tissue sections in the various staining reagents (diluted in a solution of non-jelling seaweed gelatin and a nonionizing detergent used to aid in the tissue penetration of antibody) for 48 hours at 4°C
3. Enhancement of the staining reaction by the addition of imidazole to the 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate solution
4. Inhibition of endogenous peroxidase enzyme activity by the pre-staining treatment of tissue sections with 7.5% H_2O_2, 2.8% periodic acid and 0.02% potassium borohydride (the periodic acid-borohydride method)

Formalin fixation of tissues in conjunction with acetone dehydration, and low melting point paraffin embedding was a satisfactory method of processing mouse brain for the localization of BTV antigen with the modified ABC staining technique. The method also proved satisfactory for conventional hemotoxylin and eosin (H & E) staining.

Increasing the incubation time in primary antiserum allowed for a greater number of high affinity antibody molecules to become bound to the BTV antigen in the tissue section. These more avid antibody molecules were less likely to be washed away during the subsequent steps of the

procedure, thereby increasing the sensitivity of the assay.

Inhibition of endogenous peroxide enzyme activity in the tissues to be assayed was important. Should this enzyme activity not be blocked prior to the addition of the substrate chromagen, nonspecific staining often occurred which interfered with intepretation of results. Such nonspecific staining could lead to false positive results. The present study demonstrated that the periodic acid-borohydride method sufficiently inhibited endogenous peroxidase enzyme activity in the mouse tissues assayed.

With the above modification, the presence of BTV antigen in previously inoculated suckling mouse brains was adequately demonstrated with the avidin/biotin-enhanced immunoperoxidase staining technique.

The 2 immunological techniques, i.e., the A/B-ELISA and the modified ABC immunoperoxidase staining technique, were utilized to study the immune response of adult mice to a sequential second heterotypic BTV infection. In doing so, the immune response of adult mice to single serotype and to sequential homotypic BTV infections were also evaluated. In addition, the effects of pre-existing passively acquired anti-BTV humoral and cell mediated immunity (i.e., established by adoptive transfer of anti-BTV serum and BTV-sensitized spleen cells) on the immune response to subsequent single serotype BTV infection were investigated.

Results of the study indicated that C57Bl/6 mice responded with a low antibody titer to single inoculation of BTV 11. However, the immune response of this strain of mice was substantially increasd when they were sequentially inoculated with either a homotypic or a heterotypic serotype of BTV. It was notable that the antibody response to sequential heterotypic BTV infections, i.e., BTV 17 as primary and BTV 11 as secondary infecting serotypes, was greater in mice than that to sequential homotypic BTV 11 infections. This observation along with immunoabsorbent studies suggested that prior infection with 1 serotype of BTV significantly enhanced the immune response to subsequent infection by a different BTV serotype. This is in contrast to the previously held view, based largely on in vitro serum neutralization tests, that infection of an animal by 1 serotype of a virus would not alter the host response to infection by a different viral serotype.

Experiments with adoptive transfer of anti-BTV sera indicated that pre-existence of BTV antibody allowed for an/or aided the elimination of a subsequent viral infection before the induction of anti-viral antibody production could occur. The adoptive transfer of either normal or BTV 17-sensitized spleen cells had little measurable effect on the antibody response of recipient mice to subsequent BTV 11 infection.

Clinical signs of illness were not observed in any of the BTV inoculated adult mice. Routine H & E staining of various tissues from the mice (all of which were killed 14 days after the last BTV injection) revealed no recognizable morphologic alterations. Specific immunohistochemical staining of virus also was not observed in any of the paraffin embedded sections of brain, liver, spleen, or masenteric lymph node examined with the modified avidin/biotin-enhanced immunoperoxidase technique. However, specific staining was observed in all of the positive control suckling mouse brain sections and in the hearts and spleens of infection control mice through the fifth and sixth days post-BTV 11 infection.

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APPENDIX I

LIST OF RESEARCH NUMBERS WITH CORRESPONDING PATHOLOGY ACCESSION NUMBERS

Research Number	Animal Designation		Pathology Accession Number
R00558	Positive Controls		79773
R00628	Subgroup	D2	79774
R00629	Subgroup	D3	79775
R00630	Subgroup	D1	79776
R00631	Subgroup	E2	79777
R00632	Subgroup	E1	79778
R00633	Subgroup	E3	79779
R00634	Subgroup	E4	79780
R00635	Subgroup	E5	79781
R00636	Subgroup	C1	79782
R00637	Subgroup	C2	79783
R00638	Subgroup	C3	79784
R00639	Subgroup	C4	79785
R00640	Subgroup	C5	79786
R00641	Subgroup	A1	79787
R00642	Subgroup	A2	79788
R00643	Subgroup	A3	79789
R00644	Subgroup	A4	79790
R00645	Subgroup	B1	79791
R00646	Subgroup	B2	79792
R00647	Subgroup	B3	79793
R00648	Subgroup	B4	79794
R00720	Infection Controls		79795

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Tamara Dawn Witt
William Ryan Witt

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Franklin Jr. High	1962-1965
Abilene Sr. High.	1965-1968
Cisco Jr. College	1972-1973
Texas A&M University. . .	1973-1976 and 1979-1983

Degrees Awarded:

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